MATERNAL EFFECTS IN BIRDS:
EFFECTS OF NUTRITIONAL CONDITIONS ON MATERNAL REPRODUCTIVE EFFORT AND OFFSPRING PERFORMANCE

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PhD Dissertation

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CONTENTS

SUMMARY

The environment experienced by an organism at different life stages can have substantial effects on its phenotype. The environmental conditions the mother encounters can substantially influence her investment in the present reproductive attempt, and the phenotype of her offspring through the so-called maternal effects. Moreover, prenatal and early postnatal conditions experienced by offspring can interact and modulate their life histories.

The aim of this thesis is to give new insight into environmentally induced maternal effects and to test the effects of the interaction between pre- and early postnatal conditions on offspring physiology and morphology in a wild-living bird species, the great tit (*Parus major*).

In **Chapter one,** I investigated how an environmental match / mismatch between the food availability encountered by mothers during egg laying (i.e. prenatal environment) and by nestlings during early growth affected the developmental trajectories of nestlings, testing for a possible role of yolk androgens in mediating potential prenatal maternal effects. I found no evidence that nestlings performed better when experiencing matching nutritional conditions. However, being raised in the original nest by the biological parents (i.e. matching conditions) had positive effects on nestling growth and body mass. I was able to show significant interactive effects of pre- and postnatal food availability on nestling body growth, and significant effects of maternal food availability on nestling body size and mass. However, these effects were not mediated by yolk androgen deposition. The results of this experiment suggest that food-induced maternal effects can have substantial effects on fitness-related traits of the offspring by influencing their efficiency to use nutritional resources. They also highlight the necessity to take multiple environmental factors into account when studying the effects of environmental match / mismatch on offspring phenotype.

In **Chapter two** I assessed how pre- and early postnatal nutritional conditions and their match / mismatch can affect the physiological conditions, measured as oxidative status, of male and female nestling great tits. My findings revealed that the nutritional conditions experienced by mothers and by offspring during growth affect the physiological condition of nestlings in a sex-specific way. Female nestlings whose mother has not been food-supplemented, and female nestlings, which did not receive supplementary food during the postnatal period, had higher oxidative damage than male nestlings. On the other hand, no differences in the oxidative status of males and females were found when they, pre- or postnatally, received supplementary food. This results show that nutritional conditions experienced early in life, and also already before birth, influence the oxidative status of offspring in a sex-specific way, suggesting sex-specific resource-allocation strategies early in life.

In **Chapter three,** I set out to test how food availability during egg laying affects a female's condition and reproductive effort, and to test whether the eggshell pigmentation pattern signals the oxidative status of the female, as predicted by the 'sexual signaling hypothesis'. I found that the food availability experienced by females during reproduction affects their level of oxidative damage, with females experiencing higher food availability suffering of lower levels of oxidative stress. Moreover, the nutritional and oxidative condition of the females interactively modulated the allocation of antioxidants in the eggs, and female and yolk antioxidant capacity related to the pattern of eggshell pigmentation. These findings support the prediction of the 'sexual signaling hypothesis' and they shed further light on the mechanisms underlying the costs of reproduction.

ZUSAMMENFASSUNG

Die Umwelt, der ein Organismus während verschiedenen Lebensphasen ausgesetzt ist, kann seinen Phänotyp beeinflussen. Besonders die Umwelt, die die Mutter erfährt, kann einen grossen Einfluss auf ihre Investition in ihren Nachwuchs haben. Dadurch interagiert die mütterliche Umwelt mit der Umwelt ihrer Nachkommen bei der Geburt und beeinflusst so deren Lebensgeschichte.

Das Ziel dieser Arbeit ist es, neue Kenntnisse über die umweltbedingten mütterlichen Effekte zu gewinnen und die Auswirkungen der Interaktion von prä- und postnatalen Umweltbedingungen auf die Physiologie und Morphologie des Nachwuchses zu testen. Diese Fragen untersuchte ich in einer Population wilder Vögel, der Kohlmeise (*Parus major*).

In **Kapitel eins** untersuchte ich den Einfluss der Änlichkeit der Umwelt von Mutter und Nestlingen auf die Nestlingsentwicklung und testete die mögliche Funktion von umweltbedingten mütterlichen Einflüssen. Ausserdem testete ich die Rolle von Androgenen im Eigelb beim Vermitteln der maternalen Effekte. Ich fand keine Hinweise darauf, dass es Nestlingen besser geht, wenn die Ernährungsbedingungen bei Eiablage und Schlüpfen ähnlich sind. Ich fand jedoch heraus, dass es positive Auswirkungen auf Wachstum und Körpermasse hatte, wenn die Nestlinge im ursprünglichen Nest von den biologischen Eltern aufgezogen wurden (Ähnlichkeit). Es bestanden wesentliche Auswirkungen der Interaktion von prä- und postnataler Verfügbarkeit von Nahrung auf das Körperwachstum, sowie deutliche Auswirkungen der mütterlichen Nahrungsverfügbarkeit auf die Körpergröße und die Masse. Diese Effekte wurden jedoch nicht von Androgenen im Eigelb vermittelt. Die Resultate dieses Experiments zeigen, dass durch die Nahrung induzierte maternale Effekte erhebliche Auswirkungen auf Fitness-bezogene Merkmale der Nachkommen haben können. Diese Auswirkungen entstehen durch Beeinflussung der Effizienz der Nutzung von verfügbaren Ressourcen. Diese Ergebnisse zeigen auch, dass es

notwendig ist, verschiedene Umweltfaktoren zu berücksichtigen um die phänotypischen Auswirkungen der Umweltähnlichkeit / -unähnlichkeit auf die Nachkommen zu untersuchen.

Kapitel zwei ist dem Einfluss der prä- und frühen postnatalen Umweltähnlichkeit / -unähnlichkeit auf die Physiologie der Nestlinge, gemessen als deren oxidativem Stress, gewidmet. Ich konnte zeigen, dass das Nahrungsangebot von Müttern und Nachkommen die Effizienz des antioxidativen Systems in einer geschlechtsspezifischen Art und Weise beeinflusst. Weibliche Nestlinge, deren Mutter keine Zusatznahrung erhielt, sowie Nestlinge, die während der postnatalen Phase keine Zusatznahrung erhielten, wiesen eine höhere oxidative Schädigung auf als entsprechende männliche Nestlinge. Erhielten die Nestlinge oder deren Mutter allerdings Zusatznahrung während der prä- oder postnatalen Phase, konnten keine Geschlechtsunterschiede mehr festgestellt werden. Das Ergebnis dieses Experiments legt nahe, dass die Ernährungsbedingungen zu Beginn des Lebens und auch schon vor der Geburt einen geschlechtsspezifischen Einfluss auf den oxidativen Zustand der Nestlinge haben, was auf geschlechtsspezifische Strategien der Ressourcenverteilung zu Beginn des Lebens hindeutet. Diese Ergebnisse sind besonders interessant, weil sie zeigen, wie mütterliche Effekte in der frühen Phase des Lebens die individuelle Fitness des Nachwuchses beeinflussen können.

In **Kapitel drei** habe ich den Einfluss der Verfügbarkeit von Nahrung während der Eiablage auf die Verfassung und den Fortpflanzungsaufwand der Weibchen erforscht. Ich habe untersucht ob der physiologische Zustand der Mütter, gemessen als deren oxidativen Stress, die Pigmentierungsmuster der Eierschalen beeinflusst, wie es durch die ‘sexuelle Signal-Hypothese’ vorhergesagt wird. Dieses Experiment zeigte, dass das Nahrungsangebot der Vogelmütter während der Eiablage den oxidativen Stress beeinflusst. Weibchen, die ein grösseres Nahrungsangebot zur Verfügung hatten, erfuhren weniger

oxidativen Stress. Der Ernährungs- und oxidative Zustand der Weibchen sowie deren Interaktion modulierten die Allokation von Antioxidantien in die Eier. Die antioxidative Kapazität von Weibchen und Eigelb ihrerseits korrelieren mit dem Muster der Eierschalen-Pigmentation. Diese Ergebnisse stützen die Vorhersagen der ‘sexuellen Signal-Hypothese’ und sie werfen Licht auf die Mechanismen, durch welche die Kosten der Reproduktion verursacht werden.

GENERAL INTRODUCTION

RESOURCES AVAILABILITY AND LIFE HISTORIES

The phenotype of an individual is mainly shaped by three factors, namely its genes, its prenatal environment and its postnatal rearing environment. On the one hand, the genetic background determines the expression of many phenotypic traits. On the other hand, the prenatal (i.e. maternal) and early postnatal environmental conditions are determinant to define the developmental trajectories of the individual, and modulate their survival and reproductive success (Mousseau and Fox 1998; Lindström 1999; Monaghan 2008). In addition, also the environment encountered during adulthood can exercise further influence on an individual's performance (Metcalfe and Alonso-Alvarez 2010).

One of the key factors characterizing the quality of the pre- and postnatal environments, and influencing individuals' performance is the available amount of nutritional resources. On the one hand, maternal nutritional status influences her investment in the current reproductive attempt, in terms of egg quality and quantity (Bolton et al. 1992; Schaper and Visser 2013) and incubation behavior (Eikenaar et al. 2003), and, it can indirectly affect growth and physiology of offspring (Moreno et al. 2008; Vijendravarma et al. 2010; Howie et al. 2012) with long-lasting consequences (Gorman and Nager 2004). On the other hand, the early postnatal nutritional conditions modulate the developmental trajectories of offspring (Schmidt et al. 2012; Kotrschal et al. 2014), influencing a wide range of phenotypic traits (Birkhead et al. 1999; Ohlsson and Smith 2001; Mugabo et al. 2010), and, potentially affecting their survival and adult reproductive success (Richner 1992). Moreover, the consequences of the pre- and early postnatal nutritional conditions can persist for more than one generation (Lummaa and Clutton-Brock 2002; Liang et al. 2007; Burton and Metcalfe 2014).

Offspring can try to mitigate the effects of maternal and early poor nutrition with compensatory responses, such as hierarchical tissue preservation or compensatory (catch-

up) growth (Metcalfe and Monaghan 2001). However, these strategies may have short-term and / or long-term costs for the individuals (Christiansen et al. 1992; Ozanne and Hales 1999), negatively affecting their fitness.

MATERNAL EFFECTS

Maternal effects have been defined as the influence of the maternal environment, genotype or phenotype on the phenotype of the offspring (Marshall and Uller 2007; Wolf and Wade 2009), and they have been described in a wide range of taxa. For instance, in plants, maternal morphology can influence progeny dispersal and, consequently, their fitness (Donohue 1999). In seed beetles, mothers can adjust egg size according to the host plant offspring will feed on, affecting their survival perspective and growth trajectories (Fox et al. 1997). In amphibians and fish, stressful conditions experienced by the females can affect the size of the egg (Räsänen et al. 2008) or of the larvae they produce (McCormick 2006), with consequences on offspring condition. In mammals, mothers can influence a wide range of phenotypic traits of their offspring (Curno et al. 2009; Helle et al. 2013), since there is a constant connection between embryo and mother during the whole pregnancy. In birds, mothers exert the largest influence on their progeny before the egg is laid, through the choice of the nest site (Lloyd and Martin 2004), adjusting the laying time and order of eggs (Tobler et al. 2007) and modulating the amount of resources deposited into the egg (Groothuis et al. 2005). Once the egg is laid, the influence of the mothers is limited to the incubation behavior (Giraudeau et al. 2014) and, after the nestlings hatch, to the feeding effort (Garcia-Navas and Sanz 2011).

Maternal effects often act on multiple traits of the offspring phenotype and their 'adaptive' or 'maladaptive' values are context-dependent, making difficult to generalize on their positive or negative effects on the fitness of the offspring (Marshall and Uller 2007;

Uller 2008). When the environmental conditions are heterogeneous on space or time scales, mothers are expected to use cues from their own environment as 'predictors' of the environment offspring will encounter, and to modulate the phenotype of the offspring according to the 'predicted' environment ('anticipatory maternal effects'). However, when the environment varies in an unpredictable way and females are not able to detect reliable cues of the environment offspring will likely encounter, mothers are expected to produce a range of offspring phenotypes ('bet-hedging maternal effects'), so that at least one phenotype is likely to be well adapted to the local environment, and survive. Moreover, a deterioration of the environmental conditions can also lead mothers to substantially reduce their investment in the current reproduction, reducing the quality / fitness of their offspring ('selfish maternal effects'), in order to be able to reproduce in future or to ensure their own survival (Marshall and Uller 2007).

Maternal effects can similarly affect all the progeny or differently affect male and female offspring (Komdeur and Pen 2002; Wild and West 2007). In birds, mothers can adjust the occurrence of each sex depending on the laying order (Müller et al. 2005) or induce sex-related differences in the development of the embryo as a consequence of sex-specific resources allocation (Saino et al. 2003) and / or sex-specific sensitivity to maternal compounds deposited into the egg (e.g. Müller et al. 2009; Saino et al. 2011). Indeed, males and females nestlings can respond differently to a variety of egg resources, such as carotenoids (Berthouly et al. 2008; Saino et al. 2011), androgens (Müller et al. 2009) and other hormones (Love et al. 2005).

ENVIRONMENTAL MISMATCH HYPOTHESIS

In many species the environment experienced by the mother is a good approximation of the one her offspring will encounter after birth. Under these conditions, mothers can induce

phenotypic changes to their progeny in response to local stimuli, as parasite infestation (Tschirren et al. 2004), predator pressure (Coslovsky and Richner 2011), stress levels (Zimmer et al. 2013) or food availability (Cleal et al. 2007). These anticipatory maternal effects are expected to induce modifications during early ontogeny to prepare offspring to cope with conditions coinciding to those perceived by the mother (Marshall and Uller 2007). The main prediction of this hypothesis is that the offspring will perform better when the actual context encountered by them at birth and those perceived by the mother are similar, than when the maternal and offspring environmental conditions are different ('environmental mismatch hypothesis'; Monaghan 2008).

Medical studies, devoted to investigate the effects of maternal undernutrition on offspring condition, suggested that embryos might adapt to poor nutrition in early life by developing a 'thrifty phenotype' which enables their survival and prepares them to face nutrients-poor conditions later in life (Barker 1998). Nevertheless, the long-term benefits of these physiological modifications will depend on the postnatal environmental context the individuals will live in. On the one hand, if, after birth, the environment remains nutritionally poor, the organisms will be adapted to efficiently use the few resources available. On the other hand, if the postnatal environment will be plentiful of food the 'thrifty phenotype' will predispose offspring to develop cardiovascular and metabolic diseases later in life (Barker et al. 2002; Monaghan 2008).

Observational and experimental support to this hypothesis comes from studies on domesticated mammals and birds. A study on sheep showed that a mismatch between the nutritional supply of mother and offspring can lead to altered cardiovascular activity in adulthood, while no physiological alterations were observed in individuals facing prenatal and postnatal matching conditions (Cleal et al. 2007). Similarly, in chicken, individuals encountering prenatal and postnatal *ad libitum* nutrition were heavier than the ones facing

a mismatch between the maternal and rearing nutritional regime (van der Waaij et al. 2011). Despite this evidence, a study on captive zebra finches (*Taeniopygia guttata*) failed to find negative consequences of an environmental mismatch on offspring development (Krause and Naguib 2014). Yet, only few studies have tested the prediction of the 'environmental mismatch hypothesis' in natural populations (Uller et al. 2013), leaving this challenge open.

OXIDATIVE STRESS

The use of oxygen is essential for cellular metabolism, nonetheless, the redox reactions occurring during cell respiration are responsible for the production of highly reactive oxygen species (ROS), which are potentially harmful. The uncontrolled activity of ROS can damage essential cellular compounds (e.g. proteins, lipids, DNA) and provoke oxidative stress (Halliwell and Gutteridge 2007), commonly defined as the disturbance of the balance between pro-oxidants and antioxidants in favor of the former (Sies 1991).

It has been suggested that the levels of oxidative stress can be particularly pronounced during the early phases of life (Monaghan et al. 2009). The prenatal and early postnatal periods are energetically demanding periods characterized by fast growth (Monaghan 2008). In order to maintain the accelerated metabolic activities of these life stages, the organisms use an high amount of oxygen, producing high levels of ROS (Rollo et al. 1996), and depleting antioxidant compounds (Alonso-Alvarez et al. 2007; Hall et al. 2010). Hence, the ability to resist to oxidative stress during these stages of life can profoundly influence growth and survival perspectives of juveniles (Noguera et al. 2012; Losdat et al. 2013).

Oxidative stress has been suggested as a mediator of life-histories trade-offs also during adulthood (Metcalfe and Alonso-Alvarez 2010; Metcalfe and Monaghan 2013). The

ability of resisting oxidative damage has been shown to positively relate to reproductive traits, as female fecundity and sperm quality (Bize et al. 2008; Helfenstein et al. 2010), while a depletion of antioxidant defense has been associated to high reproductive effort (Wiersma et al. 2004; Travers et al. 2010).

Organisms have evolved a complex physiological machinery whose components act at different levels to avoid or limit the deleterious consequences of oxidative stress. A first line of antioxidant defense directly reacts with ROS and neutralizes them, while other antioxidant components catalyze the conversion of ROS to harmless compounds or degrade oxidized molecules (Pamplona and Costantini 2011). Many antioxidants (e.g. carotenoids, anthocyanins, vitamins) are acquired directly through the food (Catoni et al. 2008a; Catoni et al. 2008b), and other dietary components are used to synthetize important antioxidant molecules (Dringen et al. 1999; Lee et al. 2013). Hence, the efficiency of an individual's antioxidant machinery is strongly influenced by its diet (Catoni et al. 2008a; Cohen et al. 2009). Furthermore, in birds, nestlings rely mainly on maternally transferred antioxidant protection in the first weeks after hatching (Surai and Fisinin 2013), thus maternal nutritional conditions may affect not only hers but also her offspring's oxidative status.

REPRODUCTIVE INVESTMENT IN EGGS

In oviparous species, like birds, the embryos develop into the egg, outside mother's body, thus, the female modulates her reproductive investment mainly before laying, *via* the deposition of compounds into the egg. The amount of resources mothers allocate in the egg is crucial for the development and survival of the new individual. However, the egg is a sealed environment with a limited space available for storing resources, thus, egg size is the first egg trait mothers can adjust (Bernardo 1996) in order to increase the deposition of

resources and modulate offspring development. Indeed, egg size has been positively related with a number of offspring traits, from hatching success to growth rate and survival (Christians 2002; Krist 2011) and it can be regarded as a proxy of egg quality.

In addition to nutrients, females deposit a wide spectrum of compounds into the egg, and among these, yolk androgens have been suggested as a maternal tool to adjust offspring phenotype (Groothuis et al. 2005). Since the identification of yolk androgens in avian eggs (Schwabl 1993), the study of hormone-mediated maternal effects rapidly became a central line of research in biology. Testosterone (T), androstenedione (A4) and 5α-dihydrotestosterone (DHT) are the main avian androgens, formed in the maturing follicle in the female's ovary and then transferred to the yolk (Hackl et al. 2003). Androgens levels have been found to vary with environmental and maternal conditions (Verboven et al. 2003; Tschirren et al. 2004) and they are believed to have generally positive influence on offspring fitness, promoting begging behavior and posthatching growth (Schwabl 1996; Eising et al. 2001; Groothuis et al. 2005; Tschirren et al. 2005). However, they can also have immunosuppressive effects (Sockman and Schwabl 2000).

Other important resources deposited by the females into the egg are antioxidant compounds (e.g. vitamins, carotenoids, selenium). Antioxidants are mainly deposited into the yolk, in order to protect the embryo from the damaging effects of free radicals and ROS produced during growth (Surai et al. 1996; Stahl and Sies 2003). Indeed, high levels of yolk antioxidants positively influence embryo development, egg hatchability and nestling survival (Wilson 1997). The majority of the antioxidants deposited into the yolk derive directly from maternal diet (Berthouly et al. 2007), and their concentration relates to maternal circulating antioxidant levels (Blount et al. 2002; Costantini 2010), suggesting that maternal antioxidant status and nutrition may mediate female's ability to deposit antioxidants in her eggs.

It has been suggested that females could also modulate the deposition of other egg compounds, as eggshell pigments. Pigments are transported across the cells of the shell gland and deposited in the outer layer of the shell during the process of calcification (Solomon 1987). The two main avian pigments are biliverdin, pigment responsible for greenish-blue coloration, and protoporphyrin, pigment producing reddish-brown coloration (Kennedy and Vevers 1976). Although shell pigments have been known for long time (Romanoff and Romanoff 1949), their function is still largely unclear (Reynolds et al. 2009). It has been first hypothesized that cryptic eggshell colors have evolved as anti-predatory or anti-parasitic strategy (Kilner 2006). However, over the last decade two other hypotheses have built consensus amongst ecologists and evolutionary biologists. On the one hand, given the molecular properties of protoporphyrin, the 'structural function hypothesis' (FSH) (Gosler et al. 2005; Higham and Gosler 2006), suggested that pigments are deposited in the shell in order to reinforce its weakest parts and to compensate for calcium deficiency. On the other hand, because of the antioxidant properties of biliverdin the 'sexual signaling hypothesis' (SSH) (Moreno and Osorno 2003) proposed that shell pigments signal the antioxidant condition of the female to the mates, in order to elicit higher parental effort of the male. Support for one (FSH: Gosler et al. 2005; Bulla et al. 2012) or the other hypothesis (SSH: Hanley et al 2008; Sand and Garcia-Navas 2009) comes form studies in various species, leaving the door open for more investigation on the function of shell pigments.

STUDY SPECIES

Great tit (*Parus major*) is an insectivorous hole-nesting passerine widely distributed across Eurasia and North Africa (Figure 1). Caterpillars are the principal prey of great tits,

however, they can also eat spiders and moths (Török 1986; Naef-Daenzer et al. 2000). During the breeding season food is usually a limited resource (van Noordwijk et al. 1995; Naef-Daenzer et al. 2001), and low food availability have been shown to negatively affect nestlings growth (Keller and Van Noordwijk 1994) and to influence female's investment in eggs (Schaper and Visser 2013). In this species, the reproductive season starts approximately in March when males start defending territory where females choose the nest site and start building the nest (Gosler 1993). Shortly after the nest building completion, females start laying one egg per day. Great tit eggs weight approximately 1.5 grams and, as other species of the Paridae family, they have red-brown speckled shells (Figure 2). Final clutches are constituted by 7-14 eggs which are incubated for 12-15 days (Gosler 1993). Great tit are altricial birds, thus, from hatching till fledging, approximately 18-20 days, nestlings are fed by the parents.

STUDY AREAS

The experiment presented in **Chapter one** has been performed on the Island of Gotland (57° 10' N, 18° 20 'E), Sweden. The study site contains approximately 1000 nestboxes and consists of small deciduous woodlands with oak (*Quercus robur*), ash (*Fraxinus sp*.), small areas with pines (*Pinus sp*.) and meadows (Figure 3). The work presented in **Chapter two** and **three** have been carried out in Zurichbergwald a forest close to Zurich, Switzerland (47° 23' N, 8° 34' E). This site, containing 350 nestboxes, is a managed deciduous wood with mainly oak and small plots of pines, located nearby urban settlements (Figure 3).

Figure 1. Adult great tit female.

Figure 2. Great tit eggs in the nest.

Figure 3. Gotland study area (left) and Zurichbergwald study area (right).

THESIS OUTLINE

This thesis aims to contribute to the understanding of environmentally induced maternal effects, using a bird species, the great tit *Parus major*, as study system. Interestingly, despite the large number of studies regarding prenatal maternal effects, only a limited number of researches attempted to investigate the interacting effect of pre- and early postnatal conditions on the phenotype of the offspring in wild animal populations. Hence, using a supplemental feeding approach I tested for effects of local food availability on maternal reproductive investment and I quantified the relative importance of the nutritional conditions encountered at different stages of life on nestling performance.

Birds give a perfect framework to study maternal effects. The embryo develops outside the body of the mother, restricting maternal intervention to the prelaying phases. This peculiarity facilitates the separate manipulation of the prenatal and postnatal environments, and, using the well-established cross-fostering technique, it allows to disentangle the prenatal (maternal), postnatal (environmental) and interactive effects of environmental conditions on the phenotype of the offspring. Moreover, the use of nestboxes allows a direct manipulation and control of the environmental conditions experienced by the birds during the whole reproductive period.

In **Chapter one,** I investigated whether the food availability experienced by females during the egg laying period influenced their reproductive investment and I tested the relative importance and the interactive effects of prenatal and early postnatal nutritional conditions on the early development of nestlings.

In **Chapter two,** I explored how pre- and early postnatal nutritional conditions act and interact to modulate the antioxidant defense and oxidative stress levels of male and female nestlings.

In **Chapter three,** I tested how food availability during egg laying affects females's oxidative balance and in turn the deposition of antioxidants into the egg. Moreover, I investigated how the antioxidant status of the female and her eggs relate to the pigmenation patterns of the eggshells.

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CHAPTER ONE

INTERACTIONS BETWEEN PRENATAL MATERNAL EFFECTS AND POSTHATCHING CONDITIONS IN A WILD BIRD POPULATION

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ABSTRACT

Resources and cues provided by the mother before birth are important mediators of developmental plasticity. It has been suggested that the adaptive value of such prenatal maternal effects may depend on the environment encountered by the offspring after birth, and that offspring may perform better when environmental conditions encountered by the mother and the offspring match, than when a mismatch occurs. Here, we test how prenatal maternal effects and postnatal conditions interact in influencing offspring growth and development in wild-living great tits (*Parus major)* by manipulating food availability experienced by the mother before egg laying, partially cross-fostering nestlings between nests, and manipulating food availability after hatching. We observed significant interaction effects between pre- and postnatal food conditions. Non-supplemented nestlings reached a similar fledging mass, a trait closely linked to postfledging survival, as food-supplemented nestlings when their biological mother had received extra food during egg laying. It shows that prenatal maternal investment can compensate for growth-limiting conditions after hatching. This effect was sex specific, with daughters benefiting more than sons. Furthermore, food-supplemented nestlings grew largest when their biological mother had not received extra food during egg laying, suggesting that offspring were primed prenatally, possibly through differential egg composition, to use resources more efficiently. However, we found no evidence that offspring performed generally better when pre- and postnatal food conditions matched than when a mismatch occurred. Our results demonstrate the importance of considering the postnatal environment when testing for the ecological and evolutionary consequences of prenatal maternal effects in natural populations.

INTRODUCTION

In most species, it is the mother who provides the first environment an individual encounters in its life, even before it is born. This prenatal environment can have significant and long-lasting effects on an individual's morphology, physiology, and behavior, and is thereby an important determinant of individual variation in fitness (Mousseau and Fox 1998). Key mediators of such prenatal maternal effects are resources (e.g. nutrients, Georges et al. 1995; Christians 2002) and developmental cues (e.g. hormones, Groothuis et al. 2005) mothers pass on to their offspring during early development. The quality and quantity of maternal components transferred to the offspring depends, at least partly, on the environment mothers encounter before and during reproduction (e.g. Gil et al. 1999; Tschirren et al. 2004; Fontaine and Martin 2006; Crean and Marshall 2008). Thereby they allow mothers to convey information about local environmental condition to the developing young.

Such environmentally induced maternal effects are assumed to have evolved as an adaptation to heterogeneous, but predictable environments (Mousseau and Fox 1998; Agrawal et al. 1999; Galloway and Etterson 2007). In many species, young (and especially unborn) individuals have a far more limited ability to assess current and predict future environmental conditions than their mothers. Maternal cues that adaptively guide offspring developmental trajectories, and thereby help the offspring to cope better with the environment they will encounter, will therefore be favored by natural selection ('anticipatory maternal effects', Marshall and Uller 2007). However, phenotypic plasticity is costly and not unlimited (DeWitt et al. 1998), and organizational effects during early development are often irreversible (Hales and Barker 2001). Consequently, transgenerational programming can be selectively neutral, or may even become maladaptive, if environmental conditions change, and a mismatch between maternal and

offspring environments occurs (Hales and Barker 2001; Rickard and Lummaa 2007; Wells 2007; Monaghan 2008).

In line with the hypothesis that the adaptive value of maternal effects depends on the stability or predictability of the environment, experimental studies demonstrated that a mismatch between the conditions experienced by mothers during reproduction and the conditions experienced by the offspring after birth can affect offspring performance. In American bellflower (*Campanula americana*), for example, offspring achieve higher fitness if they are grown in the same light environment as their mother, compared with plants grown in a mismatched environment (Galloway and Etterson 2007). Similarly, in sheep (*Ovis aries*) a mismatch between pre- and postnatal nutritional conditions leads to health problems in offspring, whereas no such effects are observed when pre- and postnatal food conditions match (Cleal et al. 2007). Finally, in canaries (*Serinus canaria domestica*), mothers modify their offspring's posthatching food demand, likely through differential allocation of androgens to the eggs, to match their own provisioning capacity (Hinde et al. 2009). However, despite these examples, a recent meta-analysis revealed that evidence for anticipatory maternal effects remains surprisingly weak, and that few studies have experimentally tested the 'matching environment hypothesis' in natural vertebrate populations (Uller et al. 2013).

Whereas under the 'matching environment hypothesis' we would predict that offspring perform better when pre- and postnatal conditions match than when a mismatch occurs, under alternative scenarios, prenatal condition might affect offspring performance independent of the environment encountered after hatching (Marshall and Uller 2007). For example, beneficial conditions experienced by mothers before and during reproduction might allow them to transfer a higher quality or larger quantity of limited resources (e.g. nutrients) to the developing young. This may boost offspring performance independent of

the environment encountered after birth ('silver spoon effect', Grafen 1988; Lindström 1999). Furthermore, in cooperatively breeding birds, it has been found that females encountering favorable conditions during reproduction reduce (rather than increase) their reproductive investment in anticipation of the possibility for compensation by other family members during the post-hatching period (Russell et al. 2007). Under these two alternative scenarios, prenatal maternal effects may have long-lasting effects on offspring performance, but these effects will not depend on a match or mismatch between pre- and post-natal condition.

Here we investigated how food-mediated prenatal maternal effects and postnatal conditions interact in shaping offspring growth and development in wild-living great tits (*Parus major*). We focused on food availability, rather than other ecological factors, because previous work on captive animals (Cleal et al. 2007; Hinde et al. 2009; van der Waaij et al. 2011) and humans (Hales and Barker 2001; Gluckman et al. 2008) suggested a particularly important role of interactions between food-mediated prenatal maternal effects and postnatal nutritional conditions in creating mismatch effects. Birds are particularly suited to investigate effects of the prenatal environment and its interaction with postnatal conditions on offspring performance in the wild because the embryo development takes place outside of the mother's body, facilitating the measurement of prenatal factors (e.g. Groothuis et al. 2005). Furthermore, the prenatal environment (i.e. egg size and composition) can easily be separated from postnatal conditions by cross-fostering nestlings between nests. Yet, work with wild-living birds also puts some limitations on the traits that can be measured. For example, we here focused on fledging mass and size as fitness proxies (Tinbergen and Boerlijst 1990; Both et al. 1999; Naef-Daenzer et al. 2001), but could not measure physiological traits that were found to be affected by nutritional

mismatches in humans studies (Hales and Barker 2001; Rickard and Lummaa 2007; Wells 2007).

In our study, we experimentally manipulated food availability encountered by mothers during egg laying and food availability during the rearing period in a 2 x 2 design. We partially cross-fostered nestlings between nests, which allowed us to disentangle effects of the pre- and postnatal treatments. The cross-fostering also ensured that half of the biological siblings and half of the nestmates of each rearing nest experienced a match between prenatal and posthatching conditions, whereas the other half experienced a mismatch. We tested 1) if food-mediated prenatal maternal effects have consequences for offspring growth and development after hatching, 2) if and how prenatal and posthatching conditions interact, and 3) if offspring perform better, in terms of early growth, if pre- and postnatal food conditions match than when a mismatch occurs. Finally, we measured egg size and yolk androgen concentrations to test if they mediate potential food-mediated prenatal maternal effects on offspring traits (as found in Christians 2002; Verboven et al. 2003; Gasparini et al. 2007; Hinde et al. 2009).

MATERIALS AND METHODS

Study species and experimental protocol

The study was conducted between April and June 2011 in a nestbox-breeding population of great tits (*P. major*) on the island of Gotland, Sweden. Nestboxes were checked regularly to monitor the progress of nest building. After the birds had started to build their nest, we experimentally manipulated the food availability experienced by the female before and during egg laying by providing extra food in half of the nestboxes. To this end, we attached a small plastic cup on the inside wall of all nestboxes and, after the birds had

started to build their nest, alternately assigned nests to the prelaying food supplementation (pre-F) or the control group (pre-NF). Pre-F nests received a food supplementation of 15g of maggots (*Sarcophaga* spp.) placed in the plastic cup every other day until the clutch was completed. The pre-NF nests were visited and treated as the pre-F nests, but no food was added to their plastic cup. The treatment started 4.6 ± 0.4 days before the first egg was laid. After the clutch was completed, the food supplementation stopped and females incubated their eggs without receiving extra food.

To create a match or mismatch between conditions experienced during egg laying and conditions experienced during the rearing period, and to control for potential carry-over effects of the prelaying treatment on parental provisioning after hatching, we carried out a partial cross-fostering one day after hatching (day 1) between a pre-F and a pre-NF nest with the same hatching date (N = 52 dyads). For the cross-fostering, nestlings were weighed and ranked according to their mass in their original nest. The heaviest nestling was randomly assigned to stay in the nest of origin or to be moved to the foster nest. Cross-foster treatment (stay or go) was then alternated through the mass-based rank list. This procedure ensured that there were no initial weight differences between the two broods of a cross-foster dyad after cross-fostering, or between cross-fostered and non-cross-fostered siblings raised in the original or a foster nest (see Results for details). For identification, nestlings were marked individually by clipping down feathers. During the transport between nests (mean transport time ± 1 standard deviation [SD]: 14 ± 7 min), nestlings were kept warm in a padded box to minimize potential stress. Nestlings that remained in the nest of origin were handled in the same way and removed from their nestbox for a similar duration as cross-fostered siblings to ensure that the treatment of the two cross-foster groups was as similar as possible.

After cross-fostering, one brood of each cross-foster pair was assigned to the posthatching food supplementation group (post-F, N = 52 broods), whereas the other received no extra food during the rearing period (post-NF, N = 52 broods). We alternated if the pre-F nest of a cross-foster dyad was assigned to the post-F or post-NF group. As for the prelaying treatment, post-F nests received 15g of maggots (*Sarcophaga* spp.) placed in the plastic cup inside the nestbox every other day from cross-fostering (day 1) until day 13 posthatching. The post-NF nests were visited and treated as the post-F nests, but no food was added to their plastic cup.

Food is a limited resource for great tits during reproduction (Van Noordwijk et al. 1995; Naef-Daenzer et al. 2001; Thomas et al. 2001). We can, therefore, assume that non-supplemented broods experienced harsher conditions than food-supplemented broods. The faster growth and higher body mass of nestlings raised in food-supplemented nests in this (see Results for details) and a previous study (Tschirren et al. 2007a) is in line with this assumption. Video observation during this previous study confirmed that parents feed the provided maggots to the nestlings (BT, personal observation). However, we cannot exclude the possibility that parents ate a part of the maggots themselves, and that nestlings benefited indirectly, for example, through a higher provisioning of other than the supplemented food by well-fed parents. Although we did not film clutches during egg laying, the significant effect of the prelaying food supplementation on nestling traits (see Results for details) demonstrates that the experimental treatment was effective in influencing maternal egg provisioning. Because nestlings were cross-fostered, we can exclude the possibility that these prelaying effects were due to carry-over effects on adult food provisioning after hatching.

Nestling measures

We measured nestling body mass before cross-fostering (day 1, N = 790 nestlings) and on day 14, shortly before fledging (N = 584 nestlings). Additionally, we measured nestling body mass twice during the period of linear body mass gain (day 5 and day 9) to assess the growth rate during the main growth period. Growth rate was calculated as (body mass 9 – body mass 5) / 4. On day 14, we measured metatarsus length, a proxy of body size in birds, to the nearest 0.1 mm. When nestlings were 9 days old they were ringed with a numbered aluminum ring and a small blood sample (<20µl) was collected from the tarsal vein for molecular sex determination (as described in Tschirren et al. 2003). Nestling mortality between hatching and fledging was recorded. All procedures were conducted under licences from the Swedish National Board for Laboratory Animals (S-54-11) and the Bird Ringing Centre of the Swedish Museum of Natural History (Stockholm, Sweden).

Egg composition

Because effects of the prelaying food supplementation on offspring morphology and survival are most likely mediated through differential egg composition, we analyzed three egg components that have previously been shown to be important mediators of prenatal maternal effects in birds, namely, the total nutritional content of an egg (egg weight) (Christians 2002; Krist 2011), and the concentrations of maternally derived yolk androstenedione (yolk A4) and yolk testosterone (yolk T) (Schwabl 1993; Verboven et al. 2003; Groothuis et al. 2005; Gasparini et al. 2007; Hinde et al. 2009).

For each clutch, we collected the fourth egg on the day it was laid. The mean clutch size (± 1SD) in the study population was 8.5 (± 1.4) eggs. The forth egg is thus one of the

middle eggs in the laying sequence. On the same day, we weighed the egg, separated the yolk from the albumen and froze it at -20°C until hormone analysis.

In great tits, variation in egg mass, yolk A4 and yolk T concentrations is much smaller within than among clutches (among clutch variation in egg mass: 71% (B.T. unpublished data, Christians 2002), yolk A4 concentrations: 62%, yolk T concentrations: 64% (Postma et al. 2014)), and the change in yolk A4 and yolk T with laying sequence is small (Tschirren et al. 2004). The fourth egg's weight, yolk A4 and yolk T concentration is therefore representative for the prenatal conditions experienced by its siblings during embryonic development.

We analyzed the concentrations of yolk A4 and yolk T by radioimmunoassay as described in Tschirren et al. (2009). In short, the yolks were thawed and homogenized with 400 µl of distilled water. Aliquots of this yolk / water emulsion (~100 mg) were taken, weighed (to the nearest 0.1 mg), and mixed with 150 µl of distilled water and 50 µl of ^{3}H Tracer (~ 2,000 counts/min) to assess extraction efficiency. The samples were extracted twice with 2.5 ml of 70% diethyl ether / 30% petroleum ether (vol : vol) and dried under a stream of nitrogen. These extracts were then dissolved in 1 ml 70% methanol, centrifuged, and decanted. The supernatant was dried under a stream of nitrogen and re-dissolved in phosphate-buffered saline. Yolk A4 and T were measured using Diagnostic System Laboratories (Webster, TX) radioimmunoassay kits following the manufacturer's protocol. The average recovery rate was 86% (range: 77–93%) for yolk A4 and 86% (range: 77–93%) for yolk T. We corrected measured yolk A4 and yolk T concentrations (pg / mg yolk) for extraction efficiency (i.e. concentration * 100 / recovery rate). Dilution curves confirmed reliability of extraction and assay protocols. Yolks were analysed in a single assay. Intra-assay coefficient of variation was 3.4% for yolk A4 and 2.6% for yolk T. Yolk A4 and yolk T concentrations were log-transformed for the statistical analyses.

Statistical analyses

We tested whether nestling traits (growth rate, body mass, tarsus length) were affected by environmental conditions before egg laying, by the conditions experienced after hatching, and by the interaction between prenatal and posthatching conditions using general linear mixed-effect models. Prelaying treatment (pre-F or pre-NF), posthatching treatment (post-F or post-NF), nestling sex, and all two-way interactions were included as fixed factors. We also included the cross-foster state of a nestling (cross-fostered or raised in its original nest) to test for consequences of mismatches between prenatal and posthatching environments other than those induced by the food treatment (e.g. parasite-induced maternal effects; Tschirren et al. 2004; Tschirren et al. 2007b). Clutch size, hatching date, feather length, egg weight, and yolk androgen concentrations were included as covariates to test for potential effects of nestling competition, seasonal variation in food availability, differences in developmental stage or egg size and yolk androgen-mediated maternal effects on nestling traits.

Nest of origin (nested in the prelaying treatment) and nest of rearing (nested in the posthatching treatment) were included as random effects to account for the non-independence of nestlings within broods. If significant interaction effects between the prelaying and posthatching food treatment were observed (see Results for details), we performed post hoc contrasts based on least squares means to test which treatment groups differed significantly from one another. The match or mismatch of food conditions experienced by the rearing mother (rather than the nestlings) before egg laying and during nestling rearing did not affect nestling growth or development (results not shown), and was therefore not further considered in the analyses. We also used general linear models to test for effects of the prelaying food treatment on clutch size, egg weight, and yolk androgen concentrations. Laying date, clutch size, and the time between treatment start and laying

date (for egg weight and yolk androgens) and egg weigh (for yolk androgens) were included as covariates. For the analysis of survival from hatching until fledging, we ran a generalized linear mixed model with a binomial error structure, and the same factors and covariates as described above using the glmer function, part of the lme4 package (Bates et al. 2011).

For all tests, final models were obtained by removing factors and covariates with a $P > 0.1$, starting with the least significant term. Random effects as well as the prelaying and posthatching food treatments were always retained in the models. Results of the final models are presented in the Results. If non-significant results are presented for a factor or covariate of interest, F and P values before dropping the term from the model are shown. Residuals of the models were checked for heteroscedasticity and normality. All tests were two-tailed with a significance level set at $P \leq 0.05$. Sample sizes differ among tests because of nestling mortality or missing data. Statistical analyses were performed in JMP 10 (SAS Institute Inc., Cary, NC, 1989-2007) and R 2.14.1 (R Development Core Team 2011). Means ± 1 SD are presented.

RESULTS

Effects of the prelaying and posthatching food manipulation on nestling growth and development

Hatching mass

There was no significant difference in body mass one day after hatching between nestlings originating from a pre-F or pre-NF nest ($F_{1, 72.76} = 0.026$, $P = 0.873$) or between post-F and post-NF broods ($F_{1, 24.02} = 0.001$, $P = 0.988$). The interaction effect between the prelaying

and posthatching treatment on hatching mass was non-significant ($F_{1,\ 77.89} = 0.103$, $P = 0.749$).

Growth

There was no interaction effect between the prelaying and posthatching food treatment on the rate of body mass gain during the main growth period ($F_{1,\ 560.2} = 0.108$, $P = 0.743$, Figure 1A). Furthermore, there was no main effect of the prelaying food supplementation on offspring mass gain ($F_{1,\ 54.35} = 2.771$, $P = 0.102$). However, nestlings that received extra-food during the posthatching period grew significantly faster than non-supplemented broods ($F_{1,\ 58.35} = 6.670$, $P = 0.012$, Figure 1A).

Fledging mass and size

At the end of the nestling period, we observed a significant interaction effect between the prelaying and the posthatching food supplementation on tarsus length ($F_{1,\ 296.3} = 5.911$, $P = 0.016$, Figure 1B) and body mass ($F_{1,\ 468.7} = 6.155$, $P = 0.014$, Figure 1C).

To better understand the observed interaction effects between the prelaying and post-hatching treatment, and the relative importance of prenatal maternal effects and posthatching conditions on offspring development, we performed post hoc contrasts between treatment groups. We found that if the biological mother had not received extra food during the egg laying period, food-supplemented nestlings grew larger than non-supplemented nestlings (post hoc contrast pre-NF / post-NF vs. pre-NF / post-F: $F_{1,\ 45.73} = 6.563$, $P = 0.014$, Figure 1B). The difference in body size between supplemented and non-supplemented nestlings was not significant if their mother had received extra food during egg laying (post hoc contrast pre-F / post-NF vs. pre-F / post-F: $F_{1,\ 149.3} = 1.636$, $P = 0.203$;

Figure 1B). Interestingly, food supplemented nestlings tended to be larger when their mother had not received extra food during the egg laying period than when their mother had received extra food (post hoc contrast pre-NF / post-F vs. pre-F / post-F: $F_{1, 110.8}$ = 3.327, P = 0.071, Figure 1B).

Similarly, nestlings of mothers that had not received extra food during the egg laying period were significantly heavier if they received extra food during the nestling period (post hoc contrast pre-NF / post-NF vs. pre-NF / post-F: $F_{1, 55.23}$ = 7.262, P = 0.009, Figure 1C). Again, this difference was not significant in the pre-F group (post hoc contrast pre-F / post-NF vs. pre-F / post-F: $F_{1, 135.4}$ = 0.192, P = 0.662, Figure 1C).

Nestlings of supplemented mothers raised in a non-supplemented brood reached a similar body mass as nestlings that received extra food during the nestling period (post hoc contrast pre-F / post-NF vs both post-F: $F_{1, 129}$ = 0.000, P = 0.982, Figure 1C), showing a long-lasting, compensatory effect of food-mediated maternal effects on offspring mass.

Survival

Complete nest failure was more common later in the season (χ^2_1 = 5.362, P = 0.021), but it was not significantly influenced by the posthatching food treatment (χ^2_1 = 2.377, P = 0.123). In broods where at least one nestling fledged there was no significant effect of the prelaying treatment (χ^2_1= 0.474, P = 0.491) or the posthatching treatment (χ^2_1 = 0.285, P = 0.594) on nestling mortality, and there was no significant interaction effect between the treatments (χ^2_1 = 0.651, P = 0.420).

Sex-difference in growth and development

Male nestlings were significantly heavier than female nestlings one day posthatching ($F_{1, 379} = 6.712$, $P = 0.010$). They also grew at a faster rate ($F_{1, 503.9} = 52.409$, $P < 0.001$) and reached a larger body size ($F_{1, 510.3} = 261.017$, $P < 0.001$) and a higher body mass ($F_{1, 456.4} = 96.023$, $P < 0.001$) at the end of the nestling period. Female nestlings tended to be heavier when their mother had received extra food during the egg laying period, whereas the prelaying treatment did not affect body mass of male nestlings (interaction sex x prelaying treatment: $F_{1, 454.2} = 3.496$, $P = 0.062$, Figure 2).

Effect of the prelaying treatment on clutch size and egg composition

We tested if the prelaying food treatment affected clutch size and egg composition, and if these components explained a significant amount of variation in nestling growth and development.

The prelaying food treatment did not significantly affect clutch size (pre-F: 8.1 ± 0.77 eggs, pre-NF: 8.6 ± 1.46 eggs; $F_{1, 102} = 0.482$, $P = 0.489$), egg weight (pre-F: 1.71 ± 0.15 g, pre-NF: 1.69 ± 0.12 g; $F_{1, 95} = 0.556$, $P = 0.458$), yolk A4 concentration (pre-F: 115.85 ± 33.24 pg / mg yolk, pre-NF: 106.93. ± 22.03 pg / mg yolk; $F_{1, 79} = 0.983$, $P = 0.324$) or yolk T concentration (pre-F: 77.20 ± 31.54 pg / mg yolk, pre-NF: 69.63 ± 18.44 pg / mg yolk; $F_{1, 79} = 0.756$, $P = 0.387$).

Nestlings originating from broods with larger eggs ($F_{1, 71.96} = 7.257$, $P = 0.009$), and nestlings originating from broods with lower yolk A4 concentrations ($F_{1, 69.65} = 10.175$, $P = 0.002$) were heavier one day posthatching.

Fledging mass was higher in smaller broods ($F_{1, 85.39} = 4.418$, $P = 0.039$). The weight, yolk A4 or yolk T concentration of the fourth egg of a clutch did not explain a significant

amount of variation in fledging mass (egg weight: $F_{1, 70.1} = 0.534$, $P = 0.468$; yolk A4: $F_{1, 67.1} = 0.513$, $P = 0.477$; yolk T: $F_{1, 59.9} = 0.187$, $P = 0.667$) or fledging size (egg weight: $F_{1, 73.9} = 0.649$, $P = 0.423$; yolk A4: $F_{1, 66.42} = 0.055$, $P = 0.816$; yolk T: $F_{1, 65.2} = 1.183$, $P = 0.281$).

Neither the weight ($\chi^2_1 = 0.166$, $P = 0.684$), nor the yolk T concentration ($\chi^2_1 = 0.500$, $P = 0.480$) of the fourth egg of a clutch were significantly associated with nestling survival. However, nestlings originating from broods with higher yolk A4 concentrations were significantly more likely to survive ($\chi^2_1 = 22.403$, $P < 0.001$).

Effects of cross-fostering on offspring growth and development

To test if other, non-food mediated mismatches between pre- and posthatching conditions influence offspring development, we compared the early growth, fledging mass, and size of nestlings that were raised in their original nest (non-cross-fostered) and nestlings that were raised in a foster nest (cross-fostered).

There was no significant difference in hatching mass between cross-fostered and non-cross-fostered nestlings ($F_{1, 408.7} = 0.664$, $P = 0.416$). However, nestlings that were raised in their original nest grew faster than nestlings that were raised in a foster nest ($F_{1, 495.9} = 4.951$, $P = 0.027$, Figure 3A). This difference was not explained by the time it took to move nestlings from the original nest to the foster nest during cross-fostering ($F_{1, 477} = 0.640$, $P = 0.424$).

At the end of the nestling period, nestlings that were raised in their original nest reached a higher body mass ($F_{1, 461.4} = 6.118$, $P = 0.014$, Figure 3B). Again, the time required to move nestlings from the original nest to the foster nest during cross-fostering did not explain variation in fledging mass ($F_{1, 464} = 0.050$, $P = 0.823$). No difference in fledging

size between cross-fostered and non-cross-fostered nestling was observed ($F_{1, 493.8} = 0.066$, $P = 0.797$).

DISCUSSION

We experimentally tested how food-induced prenatal and postnatal effects interact in influencing offspring growth and development in a wild bird population. Growth rate during the period of linear mass gain (between day 5 and 9 posthatching) was strongly influenced by the posthatching food treatment, demonstrating that the amount of extra food provided to the supplemented broods was sufficient to affect nestling development. Food-supplemented nestlings grew faster than controls, and this effect was independent of the prelaying food treatment. Interestingly, however, body mass at the end of the nestling period did not differ between food-supplemented nestlings and non-supplemented nestlings whose mother had received extra food during the egg laying period. It was, however, significantly lower in non-supplemented nestlings whose mothers had not received extra food. It demonstrates that prenatal maternal effects can negate growth-limiting conditions after hatching, and, given that fledging mass is strongly linked to first year survival in small passerines (Tinbergen and Boerlijst 1990; Both et al. 1999; Naef-Daenzer et al. 2001), that conditions experienced before birth can affect traits closely linked to fitness.

A similar interaction effect between the pre- and posthatching food treatment was observed on offspring tarsus length, a proxy for body size. Offspring grew largest when their mother had not received extra food during egg laying, but food was supplemented after hatching (pre-NF / post-F). Interestingly, these nestlings were even larger than food-supplemented nestlings whose mother had received extra food during the egg laying period (pre-F / post-F). This finding is in line with the results of studies in humans (Hales and

Barker 2001) and domesticated animals (George et al. 2012), and suggests that food-mediated prenatal maternal effects influence how efficiently offspring use available resources later in life.

Although we observed significant interaction effects between prelaying and posthatching food conditions on both fledging mass and size, we found no evidence that nestlings performed better when they experienced the same conditions before and after birth (i.e. a match between prelaying and posthatching nutritional conditions) than when a mismatch occurred. Thus, unlike in domesticated animals (Cleal et al. 2007; Hinde et al. 2009; van der Waaij et al. 2011) and humans (Hales and Barker 2001; Gluckman et al. 2008), short-term fluctuations in nutritional conditions do not appear to lead to detrimental mismatch effects in the offspring. However, it is important to note that we only measured short-term effects on fledging mass and size which are, although strong predictors of first year survival (Tinbergen and Boerlijst 1990; Both et al. 1999; Naef-Daenzer et al. 2001), only one aspect of performance. It would be interesting, although practically challenging given the low local recruitment rate in our population, to follow birds that experienced a match or mismatch between prenatal and posthatching conditions throughout their life to detect potential long-term costs on fitness. Furthermore, it would be interesting to measure physiological responses, which have been shown to be most strongly affected by mismatch effects in humans and laboratory animals (Cleal et al. 2007; Hales and Barker 2001; Gluckman et al. 2008).

The observed prenatal maternal effects on fledging mass and size were not mediated by egg weight (see also Nager et al. 1997; Christians 2002, but see Bolton et al. 1992), although we only measured the weight of the fourth egg and individual variation in egg weight within broods may still play a role. It suggests that changes in the composition of the eggs or differential incubation behavior by the female in response to the food

treatment caused this effect. Previous work has shown that maternal food supplementation before and during egg laying influences egg composition, and in particular the transfer of maternal yolk androgens. Food-supplemented lesser black-backed gull females (*Larus fuscus*), for example, transferred lower androgen concentrations in their eggs compared with controls (Verboven et al. 2003). Similar effects were observed in replacement clutches of black-legged kittiwake (*Rissa tridactyla*) (Gasparini et al. 2007). Furthermore, several studies have shown that exposure to high yolk androgen concentrations during embryonic development promotes posthatching growth (e.g. Schwabl 1996; Groothuis et al. 2005; Tschirren et al. 2005; but see Sockman and Schwabl 2000). Here we found no indication that the prelaying food treatment influenced maternal A4 or T transfer to the eggs, or that yolk A4 or T concentrations were associated with nestling growth, mass or size. However, nestlings originating from a brood with higher yolk A4 concentrations were significantly less likely to die during the nestling period. It indicates that the observed prenatal effects on nestling body mass were mediated by other, unmeasured components of the egg, such as carotenoids (Romano et al. 2008), immunoglobulins (Hasselquist and Nilsson 2009) or stress hormones (Meylan and Clobert 2005; Henriksen et al. 2011; Sheriff and Love 2013), or by differential incubation behavior of the female.

Interestingly, daughters tended to benefit more from food-mediated prenatal maternal effects than sons. Such sex-specific consequences of prenatal maternal effects have been described previously (e.g. Gorman and Nager 2004; Helle et al. 2013), but there is no consensus on which sex benefits from the 'silver spoon'. For example, an experimental increase of yolk androgen concentrations in Collared flycatcher (*Ficedula albicollis*) eggs increased the growth of female, but reduced the growth of male nestlings (Pitala et al. 2009), whereas the exact opposite effect was observed in Barn swallows (*Hirundo rustica*) (Saino et al. 2006). Understanding why such sex-specific responses to

the prenatal environment occur and what factors determine which sex benefits will be the focus of future work.

Although we here focused on the consequences of a match and mismatch between pre- and postnatal nutritional conditions, maternal effects are likely to arise in response to a wide range of additional environmental factors not directly measured or manipulated in this study. For example, it has been shown that nest-based ectoparasites mediate prenatal maternal effects that promote offspring defense (Heeb et al. 1998; Tschirren et al. 2007b). Because investment in immune defense is costly and only pays when infection occurs (Tschirren and Richner 2006), a mismatch between predicted and actual parasite load would have negative consequences for the offspring. Similarly, fitness costs might occur if nestlings are maladapted to their local microhabitat or climate (Lloyd and Martin 2004; Goodenough et al. 2008) or to the microbial assemblage in the nest (Goodenough and Stallwood 2012). Although it would be difficult to identify and manipulate all the environmental factors than potentially induce maternal effects, cross-fostering nestlings between nests provides an indirect way of creating an *overall* mismatch between the conditions mothers experienced during egg laying and the conditions nestlings are encountering after hatching. Interestingly, we found that nestlings grew faster and were heavier at the end of the nestling period when they were raised in their original nest (see also Berthouly et al. 2007 for similar cross-fostering effects on immune response). This effect is unlikely due to the cross-fostering procedure itself, because all nestlings were removed from the box and handled during cross-fostering. Furthermore, the time nestlings spent outside the nest during cross-fostering did not explain significant amount of variation in growth or body mass. It thereby provides indirect evidence that mismatches between the anticipated and actual environment nestlings encounter can have negative consequences,

and that environmental factors other than food availability may be the main drivers of such mismatch effects.

In conclusion, we show that food-mediated prenatal maternal effects can have important consequences for offspring traits closely linked to fitness. In particular, our results suggest that prenatal maternal cues can influence how efficiently offspring use available resources after hatching, and that a favorable prenatal environment can compensate for growth-limiting conditions after hatching. Moreover, we observed significant interaction effects between prenatal maternal effects and postnatal conditions on offspring development. Such interaction effects may at least partly explain discrepancies in the findings of maternal effect studies in natural populations, and highlight the role of directional or stochastic environmental change in mediating the consequences of maternal effects in the wild.

Figure 1. Effects of the prelaying (pre-NF / pre-F) and posthatching (post-NF / post-F) food treatment on nestling growth rate (A), nestling body size on day 14 posthatching (B) and nestling body mass on day 14 posthatching (C). Least squares means ± 1 SE are shown

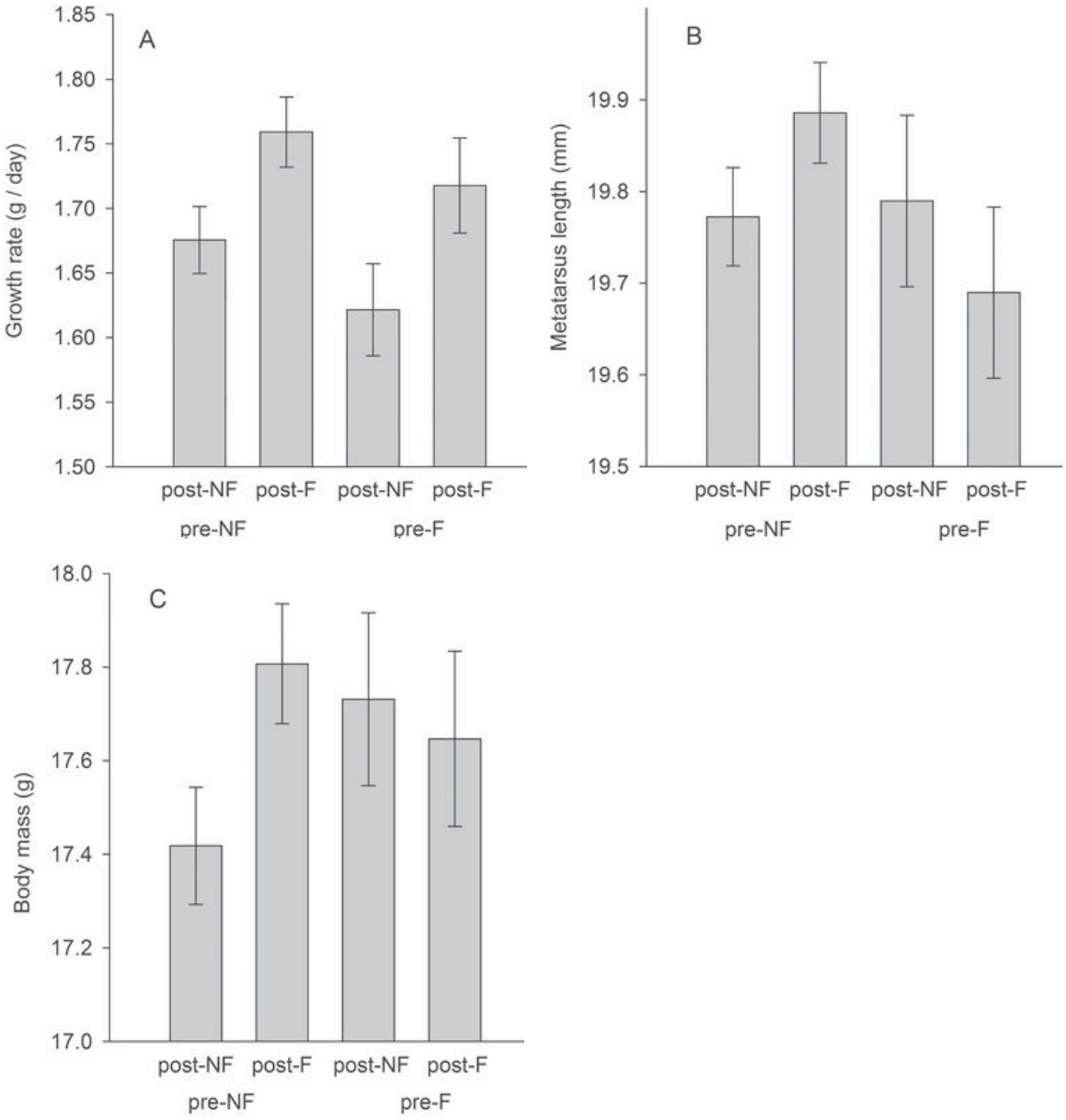


Figure 2. Effects of the prelaying food treatment (pre-NF / pre-F) on body mass of male and female nestlings on day 14 posthatching. Least squares means ± 1 SE are shown.

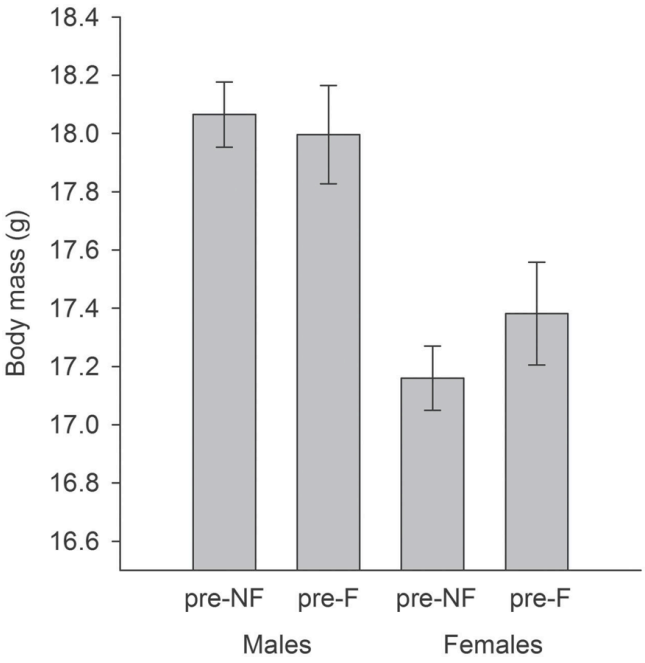
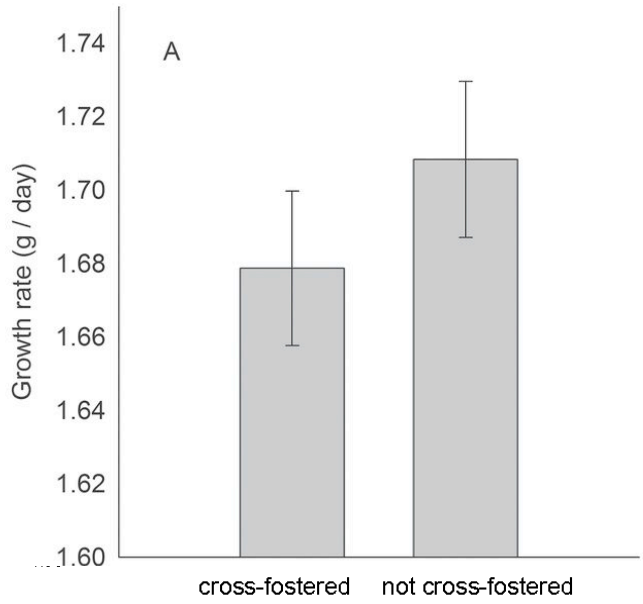
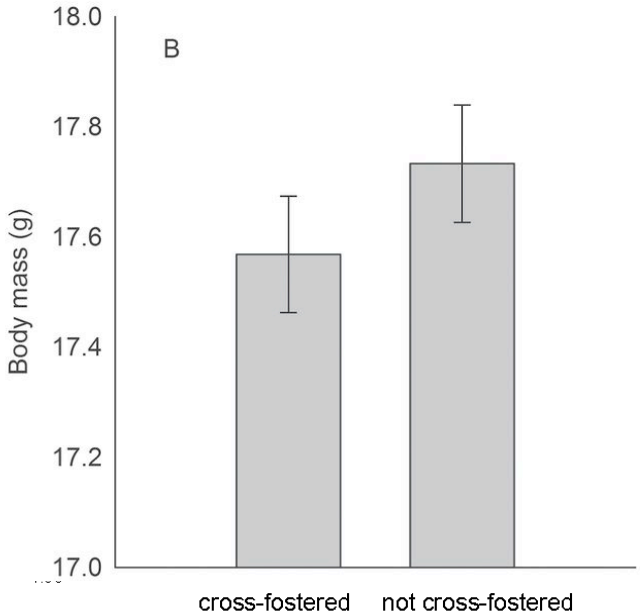


Figure 3. Growth rate (A) and nestling body mass on day 14 post-hatching (B) of nestlings that were raised in a foster nest (cross-fostered) and nestlings that were raised in their original nest (not cross-fostered). Least squares means ± 1 S.E. are shown.





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CHAPTER TWO

SEX-SPECIFIC EFFECTS OF PRENATAL AND POSTNATAL NUTRITIONAL CONDITIONS ON THE OXIDATIVE STATUS OF GREAT TIT NESTLINGS

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ABSTRACT

The early life period is characterized by fast growth and development, which can lead to high reactive oxygen species (ROS) production. Young animals thus have to balance their investment in growth versus ROS defence, and this balance is likely mediated by resource availability. Consequently resources transferred prenatally by the mother and nutritional conditions experienced shortly after birth may crucially determine the oxidative status of young animals. Here we experimentally investigated the relative importance of pre- and early postnatal nutritional conditions on the oxidative status of great tit nestlings (*Parus major*). We show that resources transferred by the mother through the egg and nutritional conditions encountered after hatching affect the oxidative status of nestling in a sex-specific way. Daughters of non-supplemented mothers, and daughters, which did not receive extra food during the early postnatal period had higher oxidative damage than sons, while no differences between sons and daughters were found when extra food was provided pre- or postnatally. No effect of the food supplementations on growth, fledging mass or tarsus length was observed, indicating that female nestlings maintained their investment in growth at the expense of ROS defence mechanisms when resources were limited. The lower priority of the antioxidant defence system for female nestlings was also evident by lower levels of specific antioxidant components. These results highlight the important role of early parental effects in shaping oxidative stress in the offspring, and show that the sensitivity to these parental effects is sex-specific.

INTRODUCTION

Already during the first stages of life, before and shortly after birth, individuals differ in the quantity and quality of resources they have available, either because of environmental variation in resource abundance and / or different maternal investment strategies (Mousseau and Fox 1998; Christians 2002). These differences in early nutritional conditions can have important and long-lasting effects on an individual's physiology, morphology and life history (Mousseau and Fox 1998; Lindström 1999; Monaghan 2008). The prenatal and early postnatal periods are characterized by fast growth and development, and are therefore nutritionally highly demanding (Keller and Van Noordwijk 1994; Naef-Daenzer and Keller 1999). At the same time, fast growth and development result in increased production of free radicals (Rollo et al. 1996), which may negatively affect fitness, both in the short- and the long-term (Costantini 2008; Metcalfe and Alonso-Alvarez 2010). Individuals thus face trade-offs and constraints in their investment in growth and the free radical scavenging system early in life (Alonso-Alvarez et al. 2007; Hall et al. 2010), and these trade-offs are likely mediated by food availability (Catoni et al. 2008a; Monaghan et al. 2009).

One consequence of high free radical production is oxidative stress, which is commonly defined as an unbalance between the production of reactive oxygen species (ROS) and antioxidant responses, in favor of the former, leading to an increase in oxidative damage (Sies 1991; Halliwell and Gutteridge 2007). ROS are constantly produced in the mitochondria as byproducts of cell respiration (Handy and Loscalzo 2012) and above certain concentrations, they become harmful and can cause cellular damage (Halliwell and Gutteridge 2007).

Organisms have evolved a complex antioxidant machinery to counter the toxicity of ROS and maintain normal physiological conditions in the cell (Halliwell and Gutteridge

2007; Pamplona and Costantini 2011). The first line of defense includes scavengers, such as thiols groups, which directly react with and neutralize oxidizing agents (Dickinson and Forman 2002; Cremers and Jakob 2013). A second line of defense includes enzymes, such as glutathione peroxidase, which catalyze the reduction of dangerous oxidizing agents (Arthur 2000).

Evidence is accumulating that the ability to mitigate the negative effects of ROS activity is an important factor underlying variation in individual fitness (Costantini 2008; Metcalfe and Alonso-Alvarez 2010). Antioxidant capacity is positively, while oxidative damage negatively related to survival (Freeman-Gallant et al. 2011; Saino et al. 2011). Furthermore recent studies showed that the ability of nestlings to cope with oxidative stress predicts their fledging success (Losdat et al. 2013) and their recruitment probability (Noguera et al. 2012), highlighting that an efficient antioxidant protection is beneficial from the first stages of life. However, investment in such an efficient oxidative protection is likely associated with costs.

Many components of the antioxidant machinery (e.g. carotenoids, vitamin E, vitamin C) are acquired directly through the food (Catoni et al. 2008a; Catoni et al. 2008b). Moreover dietary components with no specific antioxidant properties are used to build important antioxidant molecules (e.g. thiols and enzymes) (Dringen et al. 1999; Lee et al. 2013). Studies on laboratory and wild animals showed that differences in the protein content of the diet can affect the oxidative status of an individual (Feoli et al. 2006; Cohen et al. 2009; Beaulieu et al. 2010). In rats, for example, protein deficiency gave rise to higher oxidative damage and decreased antioxidant activity (Bonatto et al. 2006; Feoli et al. 2006), whereas cysteine-rich food reduced oxidative stress and increased thiols levels (Blouet et al. 2007). Furthermore a high protein content in the diet improved the antioxidant protection in juvenile white-throated sparrows (*Zonotrichia albicollis*) (Alan

and McWilliams 2013). These findings highlight the crucial role of high protein intake for the proper functioning of the antioxidant machinery.

Early in life, individuals depend mainly on maternal resources transferred prenatally (e.g. into the egg) and (in altricial species) on resources provided by the parents after birth for their protection against oxidative damage, and they start to develop their own protective machinery only after a few weeks of life (Karadas et al. 2005; Surai and Fisinin 2013). Consequently food availability encountered by the mother during egg laying and food availability encountered by the parents during the rearing period are expected to affect the offspring's antioxidant machinery. Accordingly, offspring are predicted to have highest oxidative protection and lowest oxidative damage when nutritional conditions are favorable both before and after birth.

However, higher pre- and early postnatal food availability might also trigger faster growth, which may create extra ROS. Under this scenario, favorable pre- and early postnatal food conditions could lead to higher, rather than lower oxidative stress. Thus, it is important to assess the effect and relative importance of pre- and postnatal nutritional conditions on antioxidant status and growth, respectively. Here we experimentally manipulated food availability during egg laying and food availability after hatching in a 2 x 2 design in a natural population of great tits (*Parus major*), in order to test how pre- and early postnatal conditions affect oxidative status and growth of male and female offspring.

MATERIAL AND METHODS

Study species and experimental protocol

The study was conducted between March and June 2012, in a nestbox-breeding population of great tits in Zurichbergwald, a forest close to Zurich, Switzerland (47°20'08'' N,

8°30’01’’ E). A total of 350 nestboxes (12.5cm x 12.5cm x 26.5 cm, Type Varia), placed at a distance of 100-150 meters to one another along forest roads, were regularly visited from the beginning of the breeding season onwards in order to monitor the progress of nest building and the start of egg laying. At an advanced stage of nest building, but before the first egg was laid (mean ± SD: 7.1 ± 4.7 days before the first egg was laid), we experimentally manipulated the food availability of the breeding pairs by providing additional food in half of the nestboxes. To this end, we attached a small plastic cup on the inside wall of all nestboxes. Placing the cup inside the nestbox, not visible from the outside, ensured that only the breeding pair consumed the food we provided. We randomly assigned nestboxes to the prelaying food supplementation (pre-F) or to the control group (pre-NF). Pre-F nests received a food supplementation of 15g of maggots (*Sarcophaga* spp.), which contain high levels of proteins and lipids (Hwangbo et al. 2009; Jensen et al. 2011), every other day. All maggots were eaten within two days (MG personal observation). We cannot exclude that males ate a part of the supplemented food. However, the significant effect of the pre-laying food supplementation on offspring traits (see Results for details) shows that the pre-laying treatment was effective in influencing maternal egg provisioning. Indeed, food-supplemented females tended to lay heavier eggs than non-supplemented females ($F_{1,113} = 3.553$, $P = 0.062$). No difference in clutch size between supplemented and non-supplemented females was observed ($F_{1,126} = 0.883$, $P = 0.349$). Given that nestlings were cross-fostered between nests, we can exclude the possibility that the effects of the pre-laying treatment on offspring traits were due to carry-over effects of the prelaying treatment on parental provisioning after hatching. The pre-NF nests were visited and treated as the pre-F nests, but no food was added to their plastic cup. After the clutch was completed, the food supplementation stopped and the females incubated their eggs without receiving extra food.

To manipulate the food availability experienced by the nestlings after hatching and to be able to disentangle prenatal and postnatal effects on offspring oxidative status, we carried out a complete cross-fostering (i.e. a brood swap) one day after hatching between nests with the same hatching date and a similar brood size (N = 64 dyads). Cross-fosterings were alternatingly performed between nests with the same and a different pre-laying treatment. During the transport between nests, nestlings were kept warm in a padded box to minimize potential stress. After cross-fostering, one brood of each dyad was randomly assigned to the post-hatching food supplementation group (post-F, N = 64 broods), whereas the other received no extra food during the nestling period (post-NF, N = 64 broods). The posthatching food supplementation followed the same protocol as the pre-laying treatment. Post-F nests received 15g of maggots (*Sarcophaga* spp.) every other day from day 1 until day 13 post-hatching. The post-NF broods were visited and treated as the post-F broods, but no food was added to their plastic cup.

Nestling measurements

We measured nestling body mass on day 1 posthatching (N = 756 nestlings) (hereafter 'hatching mass'), on day 3 (N = 728 nestlings), on day 9 (N = 661 nestlings) and on day 15 (hereafter 'fledging mass'), shortly before fledging (N = 517 nestlings). On day 15, we also measured tarsus length, a proxy for body size. We determined the growth rate during the linear growth period (day 3 to day 9) by calculating [(Ln (body mass day 9) – (Ln (body mass day 3)]/ 6.

Nestlings were marked individually on day 1 by clipping down feathers on their head and back. When nestlings were 9 days old, they were ringed with a numbered aluminium ring and a blood sample was collected from the tarsal vein for the analysis of oxidative stress markers and molecular sex determination (as described in Tschirren et al.

2003). The blood was kept cool until it was centrifuged (10'621x g for 10 minutes; Eppendorf 5417C centrifuge) on the same day. After centrifugation, plasma and red blood cells were separated and stored at -80° C until oxidative stress analysis (within 4 months after blood sampling).

Measurements of oxidative status

Reactive oxygen metabolites (ROMs)

We estimated the plasma concentration of reactive oxygen metabolites (ROMs) using the d-ROMs test (Diacron International, Grosseto, Italy). This colorimetric assay measures circulating hydroperoxides (Alberti et al. 2000; Buonocore et al. 2000), which are intermediate oxidative damage molecules produced by peroxidation of diverse biomolecules, including lipids and proteins (Halliwell and Gutteridge 2007). The reaction of a dilution series of cumene hydroperoxide was highly linear (range: 0 to 4.5 μM, R^2 = 0.9996; physiological values in vertebrates). Analysis followed previously published protocols (Costantini et al. 2006). In short, the plasma (8 μl) was diluted with 200 μl of a solution containing acetate buffer (pH 4.8) and an aromatic alkyl-amine as chromogen. The samples were then incubated for 75 min at 37 °C. After incubation the absorbance was read with a Thermo Scientific Multiskan Spectrum spectrometer (ThermoFisher, Vantaa, Finland) at a wavelength of 505 nm. The ROMs concentration was calculated by comparing the absorbance of the samples with a standard curve. Measurements were expressed as mM of H_2O_2 equivalents. Samples were run in duplicate. ROMs concentrations were highly repeatable within individuals (r = 0.87, $F_{106,107}$ = 14.909, P < 0.001) (Lessells and Boag 1987). The inter-assay coefficient of variation was 12.1%, and the intra-assay coefficient of variation was 4.3%.

Glutathione peroxidase activity (GPX)

The activity of glutathione peroxidase (GPX) in red blood cells was quantified using the Ransel assay (Randox Laboratories, Crumlin, UK) (Paglia and Valentine 1967). GPX is an antioxidant selenoenzyme that catalyzes the reduction of peroxides and hydroperoxides, using thiols as cofactors (Arthur 2000).The assay was performed following the manufactures' protocol. In short, the samples were diluted 1: 40 (vol : vol)with the provided dilution agent. 200 µl of reagent (glutathione 4 mmol l^{-1}; glutathione reductase ≥0.5 U l^{-1}; NADPH 0.34 mmol l^{-1}) was added to each plate well. 4 µl of the diluted samples and 8 µl cumene hydroperoxide were added and absorbance was read with a Thermo Scientific Multiskan Spectrum spectrometer (ThermoFisher, Vantaa, Finland) at 340 nm after one and three minutes. GPX activity was calculated using the formula reported in the manufacturer's instructions: [(Abs 1min-Abs 3min)/ 2]* 15'873]. GPX values were standardized by expressing them as units of GPX per mg proteins. The concentration of proteins in red blood cells was measured using the Bio-Rad Bradford Protein assay kit (Bio-Rad Laboratories, Inc., CA, USA) according to the manufacturer's instructions. Repeatability of GPX activity within individuals was high (r = 0.98, $F_{327,328}$ = 91.797, P < 0.001) (Lessells and Boag 1987). The inter-assay coefficient of variation was 12.4% and the intra-assay coefficient of variation was 5%.

Red blood cells thiols (Thiols)

We measured thiol-containing compounds in red blood cells using the -SHp test (Diacron International, Grosseto, Italy) following Costantini et al. (2011). Thiols are compounds that contain a functional group composed of a sulphur atom and a hydrogen atom (-SH). Thioredoxin and glutathione are two of the major thiols that occur in animal cells. These

molecules play important roles in antioxidant systems, acting as substrates for enzymes or directly neutralizing compounds, which cause oxidative damage, such as hydrogen peroxide (Dickinson and Forman 2002; Bindoli et al. 2008). Red blood cells were diluted 1:200 (vol : vol) with distilled water. Diluted red blood cells (12.5 µl) were added to a sulfate buffer (pH 7.6) and incubated at room temperature for 3 min. After incubation a baseline absorbance was read with a SpectraMax 340PC^{384} Microplate Reader (Molecular Devices, Sunnyvale, CA, USA) at 405 nm. Then 5 µl of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) was added and incubated at room temperature for 5 min. The absorbance was then read again at 405 nm. Concentrations were calculated using a standard solution of L-cysteine (496 mM of -SH groups). Thiols measures were expressed as µmol of –SH groups per mg proteins. Repeatability of thiols measures within individuals was high (r = 0.98, $F_{333,334}$ = 95.101, P < 0.001) (Lessells and Boag 1987). The inter-assay coefficient of variation was 6.4% and the intra-assay coefficient of variation was 5.8%.

Plasma non-enzymatic antioxidant capacity (OXY)

We measured the non-enzymatic antioxidant barrier in the plasma by colorimetric determination using the OXY- Adsorbent test (Diacron International, Grosseto, Italy). This assay quantifies the ability of the plasmatic antioxidant barrier, including exogenous and endogenous antioxidant compounds, to deal with the action of a powerful oxidant, hypochlorous acid (HOCl) (Carratelli et al. 2001). The analysis was carried out according to previously published protocols (Costantini et al. 2006). Briefly, plasma samples (2 µl) were diluted 1:100 (vol : vol) with distilled water. 2 µl diluted plasma was added to 200 µl of a titred HOCl solution and incubated at 37 °C for 10 min. After incubation, 2 µl of N,N-diethyl-*p*-phenilendiamine was added as a chromogen and the absorbance was read at a wavelength of 490 nm with a Thermo Scientific Multiskan Spectrum spectrometer

(ThermoFisher, Vantaa, Finland). Measurements are expressed as mM of neutralized HOCl. Samples were run in duplicate. Repeatability within individuals was high ($r = 0.89$, $F_{282,283} = 17.973$, $P < 0.001$) (Lessells and Boag 1987). The inter-assay coefficient of variation was 10.5% and the intra-assay coefficient of variation was 5.9%.

Statistical analyses

We used general linear mixed models (LMM) to test for effects of the pre-laying treatment, the post-hatching treatment, sex and their interactions on nestling oxidative stress biomarkers, fledging mass, tarsus length and growth rate. We also used a LMM to test for effects of the pre-laying treatment, sex and their interaction on hatching mass. Brood was included as a random factor to account for the non-independence of siblings due to shared genes and / or environmental factors. Furthermore, to test whether any of the measured oxidative stress markers were associated with nestling growth and morphology, we added the oxidative stress biomarkers as covariates in the LMMs described above. Since the stress biomarkers were not significantly correlated ($r < 0.127$, $P > 0.067$, in all cases) they were included simultaneously in the models.

The final models were obtained by removing the non-significant interactions and factors in a stepwise backward procedure, starting with the least significant interaction term. Brood (random effect), pre-laying treatment, post-hatching treatment and sex were retained in all final models. If interaction effects were significant, we performed post-hoc contrasts based on least squares means to determine which groups differed significantly from one another. Residuals of all models were checked for homoscedasticity and normality. All oxidative stress measures were log transformed for the statistical analyses. Sample sizes differ slightly among oxidative stress marker analyses because not enough

plasma was available to run all tests for all nestlings. Statistical analyses were performed in JMP 10 (Institute Inc. SAS 1989-2007).

RESULTS

Blood oxidative status of nestlings

The pre-laying food supplementation affected the levels of oxidative damage (ROMs) in a sex-specific way (Table 1). Daughters had higher ROMs levels than sons if their mother had not received extra-food during the egg laying period (post-hoc contrast: $F_{1,175.5}$ = 7.569, P = 0.007; Figure 1A). No such sex-difference was observed in offspring of supplemented mothers (post hoc contrast: $F_{1,183.7}$ = 2.678, P = 0.103; Figure 1A). Furthermore daughters of non-supplemented mothers had higher ROMs levels than daughters of supplemented mothers (post hoc contrast: $F_{1,173.7}$ = 9.772, P = 0.002; Figure 1A), but no differences in ROMs levels in male offspring from supplemented and non-supplemented mothers were observed (post hoc contrast: $F_{1,146.2}$ = 0.048, P = 0.827; Figure 1A).

A similar sex difference in ROMs concentrations was found in relation to the post-hatching treatment (Table 1). Female nestlings had higher concentrations of ROMs than male nestlings when they did not receive any extra-food during the rearing period (post hoc contrast: $F_{1,173.7}$ = 3.851, P = 0.051; Figure 1B), whereas males and females had similar concentrations of ROMs when they received extra-food during the rearing period (post hoc contrast: $F_{1,185.6}$ = 1.161, P = 0.283; Figure 1B). Differences in ROMs concentrations between female nestlings that did or did not receive extra-food during the rearing period were non-significant (post hoc contrast $F_{1,173.2}$ = 0.843, P = 0.359; Figure 1B). Similarly, differences in ROMs concentrations between male nestlings that did or did

not receive extra-food during the rearing period were non-significant (post hoc contrast: $F_{1,147.2} = 2.376$, $P = 0.125$; Figure 1B). The two-way interaction between treatments and the three-way interaction between pre-laying treatment, post-hatching treatment and sex were not significant ($P > 0.176$ in all cases), and therefore removed from the final model.

Sex-differences were also found in the antioxidant defense markers analyzed. Female nestlings had significantly lower GPX activity than males, and this effect was independent of the food treatments ($P > 0.373$ in all cases; Table 1; Figure 3). Furthermore, there was a marginally significant interaction effect between nestling sex and the post-hatching food treatment on the concentrations of thiols (Table 1; Figure 2B). Females tended to have lower thiols concentrations than males if they received no extra-food during the posthatching period (post hoc contrast: $F_{1,270} = 3.356$, $P = 0.068$; Figure 2B), whereas no such sex difference was found in supplemented broods (post hoc contrast: $F_{1, 255.5} = 0.821$, $P = 0.366$; Figure 2B). All other interaction effects were non-significant ($P > 0.117$ in all cases: Figure 2). We did not detect any effect of the treatments, sex or their interactions on OXY levels ($P > 0.303$ in all cases; Table 1; Figure 4).

Nestling growth and morphology

The pre-laying treatment had no significant effect on hatching mass (Table 2). Furthermore, pre- and post-hatching food treatments had no significant effect on nestling growth rate or fledging mass (Table 2). However, the pre-laying treatment had a marginal effect on tarsus length (Table 2), with offspring of non-supplemented mothers having slightly longer tarsi than offspring of supplemented mothers (mean ± SE: pre-F = 19.21 ± 0.05 mm; pre-NF = 19.34 ± 0.04 mm). There was no sex difference in hatching mass, but males grew faster and were larger and heavier than females shortly before fledging (Table

2). None of the interactions between treatments and between treatments and sex were significant ($P > 0.155$ in all cases), and therefore not retained in the final models.

GPX activity was significantly associated with growth rate, with nestlings that grew faster having lower GPX activity (Table 2). None of the other oxidative stress biomarkers were significantly associated with nestling growth rate, fledging mass or tarsus length (Table 2), and they were therefore not retained in the final models.

DISCUSSION

Our study experimentally demonstrates that pre- and early postnatal nutritional conditions affect the oxidative status of nestling great tits in a sex-specific way. Daughters of non-supplemented mothers, and daughters which did not receive extra food during the early postnatal period had higher oxidative damage than sons, while no differences between sons and daughters were observed when extra food was provided pre- or postnatally. These results show that nutritional conditions experienced early in life, and in particular resources transferred by the mother through the egg, play an important role in shaping the offspring's oxidative status.

These findings are in agreement with studies on laboratory rats showing that the amount of protein individuals acquire through the diet is crucial for an efficient antioxidant defense (Feoli et al. 2006; Blouet et al. 2007), and that maternal protein malnutrition impairs the functioning of the antioxidant machinery of the offspring (Bonatto et al. 2006). Interestingly, we observed no effect of the pre- or postnatal food supplementation on nestling growth, fledging mass or tarsus length. Given that non-supplemented females had higher level of oxidative damage, this suggests that when resources are limited, (female) nestlings maintained their investment in growth at the expense of oxidative defence.

A limitation of our study is that oxidative status biomarkers were measured only once during the nestling period (on day 9 post-hatching). Therefore, we could not assess how oxidative status changed over time and how the pre- and post-hatching treatments influence this dynamics. However previous studies on birds showed that the biomarkers of blood oxidative status used in this study have a significant within-individual repeatability over periods of days to years (Costantini et al. 2007; Saino et al. 2011; DC unpublished data).

Interestingly, prenatal conditions appear to be particularly important in influencing offspring oxidative damage. Indeed, the effect of the pre-laying food treatment on levels of oxidative damage was even more pronounced than the effect of the post-hatching food treatment. It suggests that mothers differentially allocated resources into their eggs in response to the pre-laying treatment, and that these maternal resources had a strong and sex-specific effect on the offspring's oxidative status. We can exclude the possibility that the effect of the pre-laying food supplementation was due to a carry-over effect that influenced maternal provisioning after hatching, because all nestlings were cross-fostered and raised by foster parents. At the moment, we can only speculate what maternal egg component mediated the observed effects. In our experiment the food-supplementation consisted mainly of protein and fat (i.e. maggots). Thus supplemented mothers may have deposited more fat and / or proteins into their eggs, which may have boosted the ROS scavenging machinery through the enhancement of antioxidant synthesis and / or activity (Dringen et al. 1999; Lee et al. 2013). Indeed, food-supplemented females tended to lay heavier eggs.

Although we found evidence for sex differences in the antioxidant protection machinery of nestlings – females generally had a lower glutathione peroxidase (GPX) activity than males, and when no extra food was provided during the early postnatal

period, they also tended to have lower thiols levels – no effect of the pre-laying treatment on these antioxidant defence components was observed. It indicates that egg composition affected an unmeasured aspect of the antioxidant machinery, which, in turn, influenced levels of oxidative damage in daughters. Alternatively, or in addition, egg composition may have affected the nestling's cell membrane composition (e.g., quantity of unsaturated fatty acids), which is known to affect the basal production of oxidative molecules (Hulbert et al. 2007).

Studies in mammals and birds often find that males are more susceptible to harsh environmental conditions than females (reviewed in Jones et al. 2009). It has been proposed that the larger body size, and the consequently higher need of resources of males, is the main factor driving this pattern (Clutton-Brock et al. 1985). However, whereas in great tits males are slightly (2.3%) larger than females, it was the females that were more susceptible to pre- and postnatal food conditions. It might be that the larger body size placed male nestlings at a competitive advantage over access to food (see also Oddie 2000), causing the observed increase in oxidative damage in female nestlings when food was limited (e.g. because of increased begging (Noguera et al. 2010)). However, whereas a competitive advantage could explain why females had higher oxidative damage than males when receiving no extra food during the nestling period, it is unlikely to explain the sex differences in oxidative damage caused by the pre-laying food treatment. The sex-specific sensitivity to differential, food-mediated egg composition suggests that females are more constrained in the allocation of limited resources to the ROS defence machinery because of different investment priorities and life history strategies (e.g. a higher investment in immune defence (Norris and Evans 2000; Tschirren et al. 2003)), and / or sex-specific differences in embryonic development.

The higher levels of oxidative damage found in females in response to resource limitation is in contrast to the study of De Coster et al. (2012) who found that male great tit nestlings had higher levels of oxidative damage when exposed to ectoparasitic fleas. The opposite effects of these two important environmental factors (food availability (this study) vs. parasitism (De Coster et al. 2012)) on the oxidative status of male and female nestlings highlights the different sensitivities of the two sexes to environmental factors, with subsequent consequences on physiological mediators of variation in fitness (Noguera et al. 2012; Losdat et al. 2013). The higher sensitivity of female nestlings to food limitation early in life suggests that females might pay a higher price for the increasing mismatch between food availability and demand due to climate change observed in many bird species (Thomas et al. 2001; Visser et al. 2003).

Generally, we found no strong relationships between the measured markers of oxidative damage or protection and nestling growth, fledging size or mass. However, there was a negative association between GPX activity and nestling growth rate, with nestlings, which grew faster, having lower GPX activity. Glutathione peroxidase activity relies on the presence of glutathione (GSH), which can also act as a scavenger and directly react with ROS (Bindoli et al. 2008). During increased physical activity, a depletion of GSH occurs, with consequent decrease in GPX activity (Leeuwenburgh and Ji 1995). Similarly, during growth nestlings undergo high metabolic activity, which can cause an increase of ROS production (Rollo et al. 1996). Hence, in order to avoid oxidative stress, a significant portion of GSH available in the blood may be depleted when reacting with ROS, and this may trigger a down-regulation of GPX synthesis. Alternatively, it might be that during growth a decrease in GPX activity occurs because GPX is directly used for ROS detoxification.

In conclusion our study shows that nutritional conditions experienced early in life, both before and shortly after birth, influence the oxidative status of great tit nestlings in a sex-specific way, with female nestlings being more sensitive to nutritional stress than males. The physiological disadvantages of females are probably not linked to higher resource requirements, since female great tits are smaller than males. It indicates that sex-specific allocation priorities due to different life history strategies caused the observed patterns.

Table 1. Effects of the food treatments and sex on plasma oxidative damage (ROMs, mM H_2O_2 equivalents; N = 226), glutathione peroxidase activity (GPX, U GPX mg^{-1} proteins; N = 328), red blood cells thiols (Thiols, μmol of -SH groups mg^{-1} proteins; N=334), plasma non-enzymatic antioxidant capacity (OXY, mM HOCl neutralized; N = 300). Brood was included as a random factor in all analyses. The variance explained by brood is presented. * $P < 0.05$.

		F	*df*	*P*
ROMs				
	Pre-laying treatment	3.460	1,95.8	0.066
	Post-hatching treatment	0.008	1,95.7	0.931
	Sex	0.236	1,180.2	0.628
	Sex × Pre-laying treatment	9.240	1,177.7	0.003*
	Sex × Post-hatching treatment	5.038	1,177.3	0.026*
	Brood (variance explained: 45.1%)			
GPX				
	Pre-laying treatment	0.064	1,112.7	0.801
	Post-hatching treatment	2.326	1,112.7	0.130
	Sex	10.414	1,241.1	0.001*
	Brood (variance explained: 63.5%)			
Thiols				
	Pre-laying treatment	2.572	1, 110.8	0.116
	Post-hatching treatment	0.001	1,110.8	0.913
	Sex	0.517	1,263.2	0.473
	Sex × Post-hatching treatment	3.910	1,262.6	0.049*
	Brood (variance explained: 45.8%)			
OXY				
	Pre-laying treatment	0.087	1,109	0.769
	Post-hatching treatment	1.899	1.109.3	0.171
	Sex	0.265.3	1,265.3	0.633
	Brood (variance explained: 28.9%)			

Table 2. Effects of the food treatments, sex and oxidative biomarkers (ROMs, mM H_2O_2 equivalent; GPX, U GPX mg^{-1} proteins; Thiols, μmol of -SH groups mg^{-1} proteins; OXY, mM HOCl neutralized) on body mass at hatching (N = 756), body mass on day 15 (N = 517), tarsus length on day 15 (N = 517), and growth rate between day 3 and day 9 (N = 651). Brood was included as a random factor in all analyses. The variance explained by brood is presented. Oxidative stress biomarkers not retained in the final models are highlighted in italic. Values for oxidative stress biomarkers not retained in the models are those prior to removal. * $P < 0.05$.

		F	*df*	*P*
Hatching mass (g)				
	Pre-laying treatment	2.252	1,121.8	0.136
	Sex	1.501	1,608.4	0.221
	Brood (variance explained: 58.8%)			
Fledging mass (g)				
	Pre-laying treatment	0.139	1,90.1	0.709
	Post-hatching treatment	1.787	1,90.1	0.185
	Sex	56.785	1,439.5	<0.001*
	ROMs	2.426	1,162.9	0.121
	GPX	2.216	1,166.7	0.138
	Thiols	1.774	1,152.8	0.185
	OXY	0.719	1,130.4	0.399
	Brood (variance explained: 64.4%)			
Tarsus length (mm)				
	Pre-laying treatment	3.144	1,90.6	0.079
	Post-hatching treatment	0.003	1,90.6	0.954
	Sex	79.883	1,461.1	0.001*
	ROMs	1.397	1,177.7	0.239
	GPX	0.027	1,128.8	0.875
	Thiols	0.069	1,149.3	0.793
	OXY	2.888	1,236.1	0.090
	Brood (variance explained: 39.7%)			
Growth rate (g day^{-1})				
	Pre-laying treatment	0.588	1, 108.9	0.445
	Post-hatching treatment	0.008	1,109.8	0.931
	Sex	7.558	1,234.9	0.003*
	ROMs	0.002	1, 163.2	0.988
	GPX	5.001	1,311.6	0.026*
	Thiols	1.258	1,236.5	0.289
	OXY	0.779	1,220.4	0.378
	Brood (variance explained: 65.9%)			

Figure 1. Effect of the prelaying (A) and posthatching (B) food treatments on plasma oxidative damage (ROMs) in female (filled circles) and male (empty circles) great tit nestlings. The *x*-axis indicates food-supplementation (F) and control (NF) treatments. Means ± 1 SE are shown.

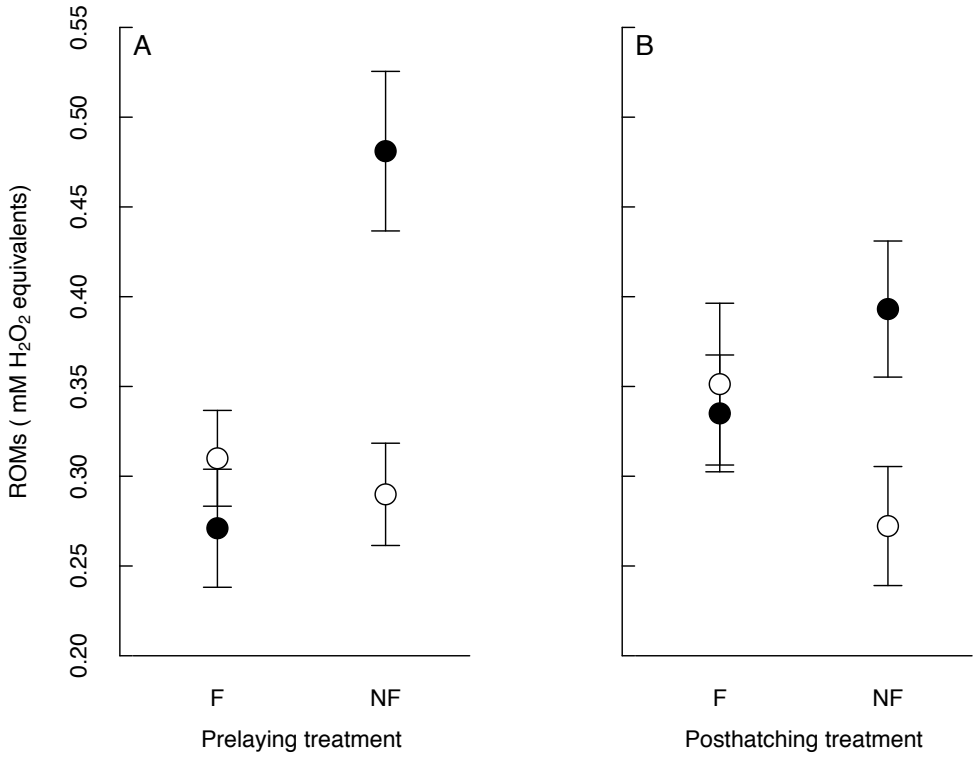


Figure 2. Effect of the pre-laying (A) and posthatching (B) food treatments on red blood cell thiols in female (filled circles) and male (empty circles) great tit nestlings. The *x*-axis indicates food-supplementation (F) and control (NF) treatments. Means ± 1 SE are shown.

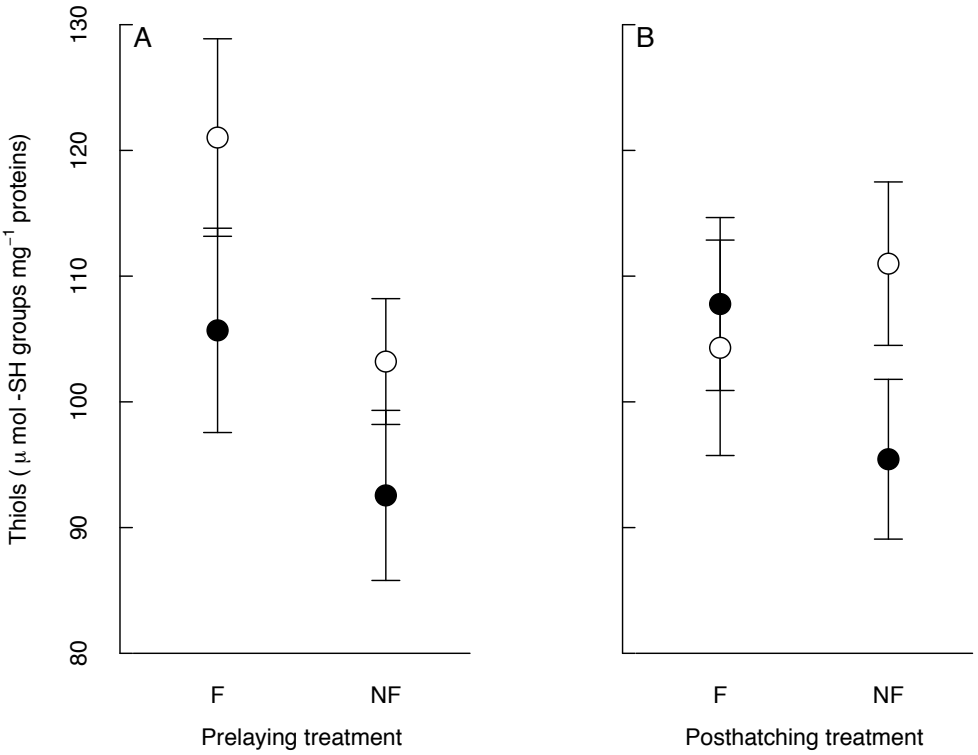


Figure 3. Effect of the prelaying (A) and posthatching (B) food treatments on glutathione peroxidase activity (GPX) in female (filled circles) and male (empty circles) great tit nestlings. The *x*-axis indicates food-supplementation (F) and control (NF) treatments. Means ± 1 SE are shown.

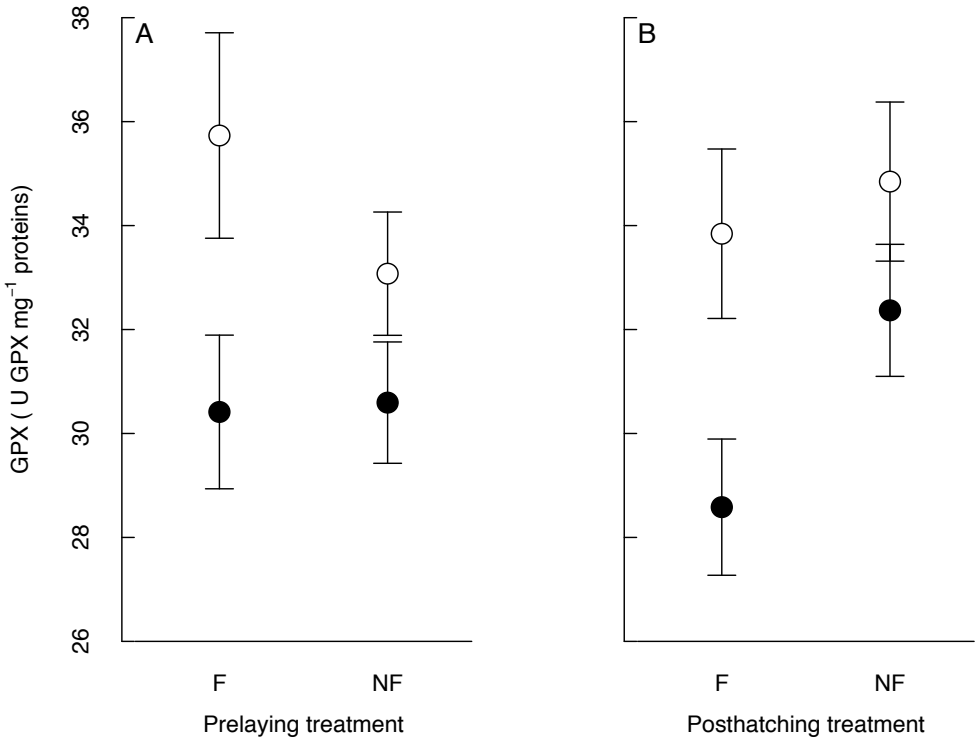
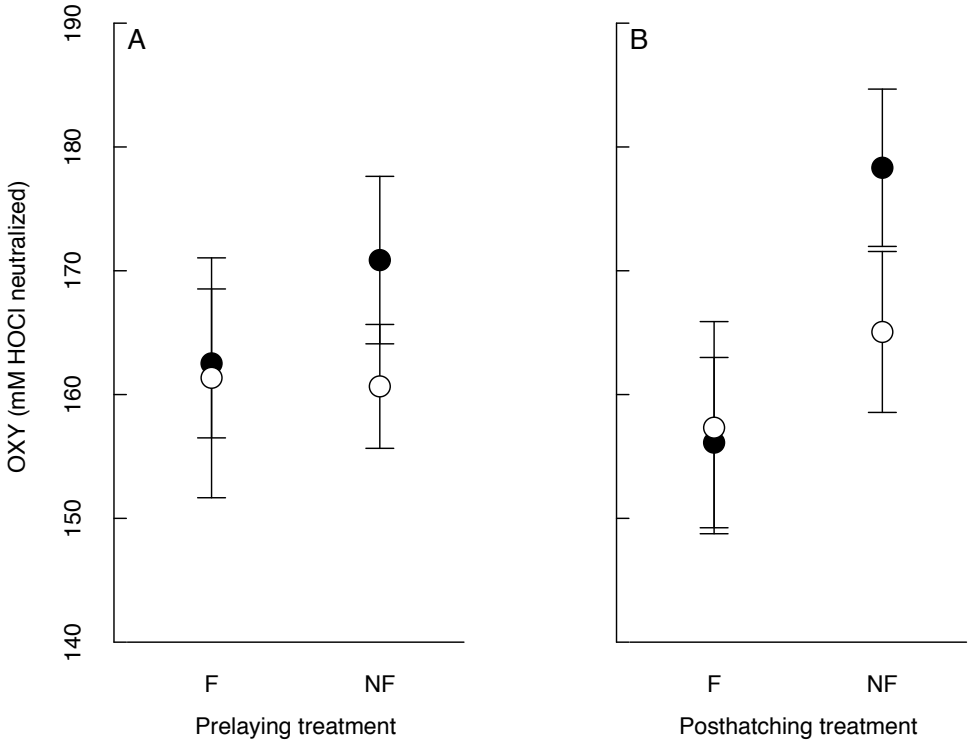


Figure 4. Effect of the prelaying (A) and posthatching (B) food treatments on non-enzymatic antioxidant barrier (OXY) in female (filled circles) and male (empty circles) great tit nestlings. The *x*-axis indicates food-supplementation (F) and control (NF) treatments. Means ± 1 SE are shown.



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CHAPTER THREE

Female oxidative status, egg antioxidant protection and eggshell pigmentation: a supplemental feeding experiment in great tits

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To be submitted

ABSTRACT

Oxidative stress has been suggested as a mechanism underlying the costs of reproduction and life-history trade offs. Reproductive activities may lead to high production of pro-oxidants, whose activity can generate oxidative damage when not countered by the individual's antioxidant defenses. Because inter-individual differences in the efficiency of the antioxidant system are influenced by an individual's diet, food availability experienced during reproduction may affect the females' antioxidant status and, in birds, their ability to transfer antioxidants into their eggs. Moreover, the female's ability to cope with oxidative stress has been suggested to influence pigment deposition in the eggshell, suggesting a possible signaling function of egg maculation. Here we performed a food supplementation experiment in a natural population of great tits (*Parus major*), in order to investigate how nutritional conditions experienced during reproduction affect the female's oxidative status and egg investment, and how maternal oxidative status and egg antioxidant protection relate to eggshell pigmentation. We show that food-supplemented females had lower oxidative damage levels (ROMs) than non-supplemented females. Furthermore, the female's ROMs levels were negatively associated with the levels of yolk antioxidant protection, and this negative association was particularly pronounced in non-supplemented females. This suggests that oxidative stress experienced during reproduction mediates the allocation of antioxidants into the egg. Moreover, we observed a positive relationship between eggshell pigment distribution and maternal and yolk antioxidant protection, suggesting that eggshell pigmentation is a signal of female quality.

INTRODUCTION

Reproduction is one of the fundamental biological processes in an individual's life, yet it is energetically and metabolically costly (Stearns 1992; Harshman and Zera 2007; Hansen et al. 2013). In oviparous species, for example, the metabolic rate of females increases by up to 27 % during egg laying (Nilsson and Råberg 2001), and both fat and protein reserves become depleted (Williams 2005). Furthermore, during incubation an up to threefold increase of the female's metabolic rate has been documented (Bryant 1997; de Heij et al. 2007; Nord et al. 2010).

The metabolic acceleration occurring during reproduction can translate into higher production of reactive oxygen species (ROS) and circulating free radicals (Alonso-Alvarez et al. 2004; Wiersma et al. 2004). The uncontrolled activity of such molecules can cause cell damage and oxidative stress (Halliwell and Gutteridge 2007), which is defined as the imbalance between pro-oxidants and antioxidant defense in favor of the former (Sies 1991). Indeed, an increase of oxidative damage, and reduction of antioxidant protection, has been observed in response to higher reproductive effort (Wiersma et al. 2004; Travers et al. 2010), suggesting oxidative stress as a cost of reproduction (Costantini 2008; Metcalfe and Alonso-Alvarez 2010; Metcalfe and Monaghan 2013).

The antioxidant system of animals acts through different lines of defense, which involve endogenous elements (e.g. thiol groups and enzymes) and exogenous compounds acquired through the food (e.g. vitamins and carotenoids) (Catoni et al. 2008a). The efficiency of the antioxidant system of an individual may thus be affected by its diet (Catoni et al. 2008a; Cohen et al. 2009), in particular by the availability of proteins. Indeed, a low-protein diet has been found to lead to increased oxidative damage and decreased antioxidant activity in both captive and wild-living animals (Feoli et al. 2006; Alan and McWilliams 2013).

In birds, the physiological and nutritional condition of the female can affect her ability to deposit antioxidants into the eggs (Blount et al. 2002; Saino et al. 2002; Berthouly et al. 2007; Török et al. 2007; Costantini 2010). Egg antioxidants provide protection to the developing embryo against the deleterious effects of free radicals produced during prenatal growth (Surai et al. 1996), and low levels of yolk antioxidants have been shown to impair embryo development (Wilson 1997). Furthermore, low antioxidant availability during embryo development can negatively affect an individual's reproductive success later in life (Surai 2002), highlighting the crucial role of maternally-derived yolk antioxidants in modulating inter-individual variation in fitness.

In birds, the shells of the eggs are characterized by a variety of colour patterns created by the deposition of two main pigments: protoporphyrin, which causes red-brown eggshell coloration, and biliverdin, which causes blue-green eggshell coloration (Kennedy and Vevers 1976). Because of the antioxidant properties of biliverdin (Kaur et al. 2003), it has been suggested that the deposition of pigments into the eggshell may signal the female's antioxidant capacity to her mate ('sexual signaling hypothesis' (SSH); Moreno and Osorno 2003). Indeed, females with a higher ability of free radicals scavenging lay eggs with more intense blue-green coloration (Hanley et al. 2008: reviewed in Reynolds et al. 2009). Protoporphyrin, on the other hand, has pro-oxidant properties (Afonso et al. 1999), and it has been suggested that an intense red-brown shell pigmentation may either signal higher oxidative tolerance of females capable of efficiently removing protoporphyrin via the shell (Sanz and Garcia-Navas 2009), or a poor physiological condition of females experiencing high levels of circulating protoporphyrin (Martinez-de la Puente et al. 2007; Duval et al. 2013). However, no study to date has directly tested for a relationship between protoporphyrin-based eggshell colours and female oxidative condition.

Here we experimentally manipulated the availability of protein-rich food shortly before and during egg laying in a natural population of great tits (*Parus major*), in order to investigate how food availability during reproduction affects female oxidative status, yolk antioxidant capacity and their relationship with eggshell pigmentation. We predict that food-supplemented females have enhanced oxidative protection and / or reduced oxidative damage, and that females with lower oxidative stress produce eggs with a higher antioxidant content. Furthermore, under the signaling hypothesis, we predict to find a relationship between female oxidative status and eggshell pigmentation.

MATERIAL AND METHODS

Field procedures

The study was carried out between March and June 2012 in a nestbox breeding population of great tits in Zurichbergwald, Switzerland (47°20'08'' N, 8°30'01'' E). The nestboxes (12.5cm x 12.5cm x 26.5 cm, Type Varia) were checked every other day from March onward to monitor the progress of nest building and the beginning of egg laying. At an advanced stage of nest building, but before the first egg was laid (mean ± 1 standard deviation (SD) = 7.1 ± 4.7 days before egg laying), we randomly assigned nestboxes to the food supplementation (F) or to the control group (NF). F-nests were supplemented with 15g of diptera larvae (*Sarcophaga* spp) every other day until the clutch was completed. The NF nests were visited and treated as the F nests, but they did not receive extra food.

In great tits the time from the beginning of ovum development to its deposition is approximately 4 days (Perrins 1996). Consequently, food-supplemented females experienced increased food availability before the formation of the first egg and during the whole egg laying period. In order to ensure that only the breeding pair had access to the

supplemented food, we placed the larvae in a plastic cup, which was attached to the inside wall of the nestbox, and not visible from the outside. All larvae were eaten within two days (MG pers. obs.). Although we cannot exclude the possibility that the male ate a part of the supplemented food, the effects of the treatment on the female's oxidative condition (see Results for details) show that females ate a substantial part of the supplemental food and that this influenced their egg provisioning.

We marked newly laid eggs with a non-toxic marker, and collected the fourth egg of each clutch on the day it was laid. On the same day, the egg was weighed, and the yolk was separated from the albumen, weighed and frozen at -80°C until analysis.

The mean clutch size (± SD) in our study population was 8.2 (± 1.3) eggs. The fourth egg is therefore one of the middle eggs of the laying sequence. In great tits, variation in the levels of yolk antioxidants is low within clutches, and yolk antioxidant concentrations do not vary with laying sequence (Isaksson et al. 2008; Remeš et al. 2011; see also Tschirren et al. 2004; Postma et al 2014 for other egg components). Therefore the antioxidant capacity of the fourth egg can be considered representative for the antioxidant capacity of the clutch.

At clutch completion, a photo of all eggs was taken. Thereto, the eggs were removed from the nest, placed on a plate, and photographed in a standardized way using a Canon EOS 1000D digital camera (Canon Inc., Tokyo, Japan) from a distance of 10 cm. Millimeter paper was photographed together with the eggs to allow for calibration.

Breeding females (F: $N = 31$; NF: $N = 39$) were caught when their nestlings were 10 days old. They were weighted with a Pesola spring balance (accuracy of 0.25 g) and a small blood sample (approximately 100 µl) was taken from their brachial vein. The blood was kept cool until centrifugation (10,621x g for 10 minutes), which occurred on the same day. After centrifugation, we separated plasma and red blood cells and stored both at -80°

C until oxidative stress analyses (see below), which was performed within 4 months after blood sampling.

Measurements of female oxidative status

Plasma non-enzymatic antioxidant capacity

We measured the females' non-enzymatic plasmatic antioxidant capacity (OXY) (F: N = 31; NF: N = 39) using the OXY- Adsorbent test (Diacron International, Grosseto, Italy) following Costantini et al. (2011). This assay quantifies the ability of a biological matrix (plasma or yolk) to oppose the oxidizing action of hypochlorous acid, one of the strongest oxidant agents in biological systems (Halliwell and Gutteridge 2007). In short, plasma (2 µl) was diluted 1:100 (vol : vol) with distilled water. Then 2 µl of the diluted plasma was added to 200 µl of a titred HOCl solution and incubated for 10 min at 37 °C. At the end of incubation, 2 µl of chromogen were added and the absorbance was read at a wavelength of 490 nm (SpectraMax 340PC^{384} Microplate Reader, Molecular Devices, Sunnyvale, CA, USA). Measurements are expressed as mM of HOCl neutralized. Samples were run in duplicate. Repeatability of OXY measures was high (r = 0.93, P < 0.001, N = 70) (Lessells and Boag 1987). The interassay coefficient of variation was 5.2% and the intrassay coefficient of variation was 3.7%.

Glutathione peroxidase activity

Glutathione peroxidase activity (GPX) in the females' red blood cells (F: N = 31; NF: N = 39) was measured with the Ransel assay (Randox Laboratories, Crumlin, UK) (Paglia and Valentine 1967) according to the manufacturer's instructions. GPX is an antioxidant enzyme which, using thiols as cofactors, catalyzes the reduction of harmful oxidant agents

(Arthur 2000). The Ransel assay assesses the ability of GPX to catalyze the neutralization of cumene hydroperoxide via oxidation of glutathione. Briefly, red blood cell samples were diluted 1: 40 (vol : vol) with the dilution agent provided with the kit. 200 µl of reagent and 8 µl of cumene hydroperoxide were added to 4 µl of the diluted sample, and, after one and three minutes, absorbance was read at a wavelength of 340 nm (ThermoFisher, Vantaa, Finland). GPX activity was calculated following the manufacturer's instructions: [(Abs 1min-Abs 3min)/ 2]* 15'873. To control for differences in red blood cell concentrations among samples, we measured the protein content using the Bio-Rad Bradford Protein assay kit (Bio-Rad Laboratories, Inc., CA, USA) according to the manufacturer's instructions. The results are expressed as units of GPX per mg proteins. Samples were run in duplicate. Repeatability of GPX activity measures was high (r = 0.97, $P < 0.001$, $N = 70$) (Lessells and Boag 1987). The interassay coefficient of variation was 9.7% and the intraassay coefficient of variation was 5.3%.

Red blood cell thiols

We measured thiol-containing compounds in the females' red blood cells (F: $N = 31$; NF: $N = 39$) using the –SHp test (Diacron International, Grosseto, Italy) according to previously published protocols (Costantini et al. 2011). Thiols are antioxidant compounds characterized by a sulfhydryl group (-SH), which reacts with, and scavenges, free radicals. Glutathione is one of the major thiols occurring in animal cells (Bindoli et al. 2008). For the assay, red blood cells samples were diluted 1:200 (vol : vol) with distilled water. 12.5 µl of diluted red blood cells were added to a sulfate buffer (pH 7.6) and let incubate for 3 min at room temperature. After incubation, absorbance was read at 405 nm (ThermoFisher, Vantaa, Finland). 5 µl of chromogen (purchased with the kit) was added and samples were incubated for 5 min at room temperature. At the end of incubation, the absorbance was

read again at 405 nm (ThermoFisher, Vantaa, Finland). Results were expressed as μmol l^{-1} of –SH groups per mg proteins. Samples were run in duplicate. Red blood thiols measures were highly repeatable (r = 0.97, P < 0.001, N = 70) (Lessells and Boag 1987). The interassay coefficient of variation was 6.7% and the intraassay coefficient of variation was 6.4%.

Because some of the markers of female antioxidant capacity were significantly correlated (GPX / Thiols: r = 0.167, P = 0.041, N = 70; GPX / OXY: r = 0.185, P = 0.023, N = 70; Thiols / OXY: r = -0.084, P = 0.303, N = 70), we performed a Principal Component Analysis and used the first two principle components (antioxidant capacity PC1 and PC2) in the statistical analyses. Oxidative stress biomarkers were log transformed before performing the Principal Component Analysis. The first principal component (antioxidant capacity PC1) explained 41.8 % of the variation in female antioxidant status. Antioxidant capacity PC1 describes the component of the female's antioxidant system involving thiols and GPX activity (enzymatic-thiolic component) (Loadings: GPX: 0.793, Thiols: 0.792, OXY: 0.305). The second principal component (antioxidant capacity PC2) explained further 34.4 % of the variation in female antioxidant status. Antioxidant capacity PC2 describes the non-enzymatic plasmatic antioxidant capacity (Loadings: GPX: 0.222, Thiols: -0.234, OXY: 0.963).

Oxidative damage

We estimated the females' plasma concentration of oxidative damage (F: N = 31; NF: N = 39) using the colorimetric dROMs test (Diacron International, Grosseto, Italy). This assay measures intermediate oxidative damage molecules (mostly hydroperoxides; Alberti et al. 2000). The assay was performed following previously published protocols (Costantini et al. 2011). In short, plasma (8μl) was diluted with 200μl of an acetate buffer (pH 4.8) and

an aromatic alkyl-amine (chromogen). The samples were incubated for 75 min at 37 °C. At the end of the incubation, the absorbance was read at a wavelength of 505 nm (ThermoFisher, Vantaa, Finland). Results are expressed as mM of H_2O_2 equivalents. Samples were run in duplicate. The repeatability of ROMs measures was high ($r = 0.98$, $P < 0.001$, $N = 70$) (Lessells and Boag 1987). The interassay coefficient of variation was 10.4%, and the intraassay coefficient of variation was 5.4%. Oxidative damage measures (ROMs) were log transformed prior to statistical analyses.

Yolk antioxidant capacity

We measured the yolk antioxidant capacity (F: $N = 14$, NF: $N = 11$) using the OXY-Adsorbent test (Diacron International, Grosseto, Italy) following previously published protocols (Costantini 2010). In short, we extracted the antioxidants in the yolk by diluting an aliquot of yolk (60 mg) with 5 ml of distilled water, and vortexing and sonicating this solution for 10 min. The dilution of yolk with water produces an emulsion in which all yolk antioxidants are embedded. We then performed the same procedure for a second aliquot of yolk, to which we added 15 µl of a solution of known concentration of OXY standard. The difference in concentration between the second yolk aliquot and the OXY standard was divided by the concentration of the first yolk aliquot to determine extraction efficiency, which was (mean ± SD) 95 ± 1.9%. The supernatant of the yolk solution was diluted 1 : 4 (vol : vol) with distilled water. 2 µl of the diluted yolk solution was then incubated with 200 µl of titred HOCl for 10 mins at 37°C. At the end of the incubation, 2 µl of chromogen (purchased with the kit) was added, and the absorbance was spectrophotometrically read at a wavelength of 490 nm (SpectraMax 340PC^{384} Microplate Reader, Molecular Devices, Sunnyvale, CA, USA). Measurements are expressed as µM of HOCl neutralized per mg yolk. Samples were run in duplicate. Yolk OXY measures were

highly repeatable (r = 0.98, P < 0.001, N = 25) (Lessells and Boag 1987). The interassay coefficient of variation was 7.4%, and the intraassay coefficient of variation was 5.5%. Yolk OXY values were log transformed before performing statistical analyses. Since it was not possible perform the OXY assay on some eggs, the yolk OXY sample size is lower than total number of eggs collected.

Eggshell pigmentation

Eggshell pigmentation (F: N = 209 eggs of 31 clutches, NF: N = 271 eggs of 39 clutches) was scored using the criteria described in Gosler (2000). Pigment intensity (I) was scored from 1 (pale pigments) to 5 (dark pigments). Pigment distribution (D) was scored from 1 (pigments concentrated on one part of the egg) to 5 (pigments evenly distributed). Spot size (S) was scored form 1 (small spots) to 3 (big spots). Zero scores were not assigned. All eggs were scored by the same person (MG). In order to calculate repeatability of eggshell pigmentation scores, a subset of eggs was scored twice. Repeatability was I: r = 0.67 P < 0.001, N = 130; D: r = 0.72, P < 0.001, N = 130; S: r = 0.51, P < 0.001, N = 130 (Lessells and Boag 1987). Pigmentation scores were correlated (Spearman rank order correlation: I/S: ρ = 0.300, P < 0.001 N = 480; I/D: ρ = -0.184, P < 0.001 N = 480; S/D: ρ = 0.059, P = 0.196; N = 480). Therefore, we calculated mean I, D and S per clutch and performed a Principal Component Analysis. The first two principal components explained 77% of the variation in eggshell pigmentation pattern (egg pigmentation PC1: 42.3%; PC2: 34.7%). Egg pigmentation PC1 describes the variation in intensity and spot size (hereafter 'darkness') (Loadings: I: 0.812, D: -0.145, S: 0.767). Eggs with more intense pigments and bigger spots had higher egg pigmentation PC1 values. Egg pigmentation PC2 describes the variation of the distribution of spots on the shell (hereafter 'spread') (Loadings: I: -0.161, D: 0.944, S: 0.349). Eggs with more evenly distributed spots on the shell had higher PC2

values.

In order to validate the qualitative method used to score pigmentation pattern (see above), we quantified eggshell pigment cover of one random egg per clutch (the one in the center of each picture) with the software ImageJ (Rasband 2004) and estimated the relationship between qualitative and quantitative maculation scores. The images were first converted to greyscale and the area occupied by black pixels was measured (pigmented area; black / white threshold = 120 brightness units). In order to control for egg size, the total egg area was also measured. The pigment cover was calculated as the ratio between pigmented area and total egg area. Before measuring the pigmentation pattern, the amount of light in each picture was standardized using the software Photoshop (Adobe System; image adjustments settings: shadows and highlights set to 100%). In order to calculate repeatability, pigment cover was measured twice in a subset of egg images ($r = 0.82$, $P < 0.001$, $N = 44$) (Lessells and Boag 1987). Pigmentation darkness (PC1) and spread (PC2) were positively correlated with eggshell pigment cover measured in ImageJ (Spearman rank order correlation: PC1: $\rho = 0.353$, $P = 0.001$, $N = 70$; PC2: $\rho = 0.179$, $P = 0.034$, $N = 70$). Darkness (PC1) and spread (PC2) are therefore informative descriptors of eggshell maculation (see also Brulez et al. 2014).

Statistical analyses

Firstly, we used general linear models to test for effects of the food treatment on female antioxidant capacity PC1 and PC2, female oxidative damage, egg size (egg mass and yolk mass), and clutch size.

Secondly, we fitted a general linear model to assess whether food treatment, female antioxidant capacity PC1 and PC2, female oxidative damage, and their two-way interactions affected yolk antioxidant capacity.

Finally, we used general linear models to test for the effects of the food treatment, female antioxidant capacity PC1 and PC2, female oxidative damage, yolk antioxidant capacity, and their two-way interactions on eggshell pigmentation (darkness (PC1) and spread (PC2)).

Final models were obtained with a stepwise backward procedure. Non-significant interactions and factors ($P > 0.05$) were removed from the models, starting with the least significant interaction term. Residuals from all the models were checked for homoscedasticity and normality. No variance inflation factors (VIF) equal to or higher than 2 were identified (Marques de Sá 2007). Analysis were performed in JMP 10 (SASInstitute 1989-2007).

RESULTS

Effect of food treatment on female oxidative status

Food-supplemented females had significantly lower levels of oxidative damage than non-supplemented females (ROMs: $F_{1,68} = 5.976$, $P = 0.017$; Figure 1). No effect of the food supplementation on the females' antioxidant capacity PC1 ($F_{1,68} = 1.079$, $P = 0.303$) or antioxidant capacity PC2 ($F_{1,68} = 1.934$, $P = 0.169$) was observed. No significant effect of the food supplementation on female body mass was found ($F_{1,68} = 1.562$, $P = 0.216$).

Effects of food treatment on yolk antioxidant status and reproductive investment

The food supplementation did not significantly affect the antioxidant capacity of the eggs ($F_{1,23} = 0.297$, $P = 0.591$), egg mass ($F_{1,63} = 0.124$, $P = 0.726$), yolk mass ($F_{1,63} = 0.421$, $P = 0.519$), or clutch size ($F_{1,68} = 2.532$, $P = 0.116$). Furthermore, there was no treatment

effect on eggshell pigmentation (darkness (PC1): $F_{1,68}$= 2.02, P = 0.160; spread (PC2): $F_{1,68}$= 0.074, P = 0.786).

Relationship between female oxidative status and yolk antioxidant status

The females' levels of oxidative damage (ROMs) were negatively related to the antioxidant capacity of their eggs ($F_{1,19}$ = 11.207, P = 0.003). Moreover, there was a significant interaction effect between the food treatment and the females' levels of oxidative damage on yolk antioxidant capacity ($F_{1,19}$ = 11.203, P = 0.003; Figure 2). The decrease of the egg antioxidant capacity with increasing ROMs levels of the mother was stronger in non-supplemented (b = -0.165), than in supplemented females (b = -0.018). No relationship between female antioxidant capacity PC1 ($F_{1,19}$ = 0.034, P = 0.855) or PC2 ($F_{1,19}$ = 1.801, P = 0.195) and egg antioxidant capacity was observed.

Relationships between eggshell pigmentation and female oxidative status and yolk antioxidant status

Eggshell pigmentation spread (PC2) was significantly positively related to female antioxidant capacity (PC1) ($F_{1,22}$ = 7.839, P = 0.010; Figure 3A). Females with a higher antioxidant capacity laid eggs with a more homogenous spot distribution. Similarly, pigmentation spread was significantly related to the antioxidant capacity of the yolk ($F_{1,22}$ = 5.592, P = 0.027, Figure 3B). Eggs with a higher yolk antioxidant capacity had a more homogenous shell spot distribution. No relationships between eggshell pigmentation spread (PC2) and female antioxidant capacity PC2 ($F_{1,21}$ = 2.057, P = 0.167) or female oxidative damage ($F_{1,19}$ = 0.802, P = 0.382) were observed.

No relationships between female antioxidant capacity (PC1: $F_{1,67}$ = 0.144, P = 0.706; PC2: $F_{1,20}$ = 0.159, P = 0.694), female oxidative damage ($F_{1,66}$ = 0.002, P = 0.964), or yolk antioxidant capacity ($F_{1,21}$ = 0.757, P = 0.394) and pigmentation darkness (PC1) were observed.

None of the interactions between food treatment and female or egg traits (P > 0.095 in all cases) significantly affected pigmentation spread (PC2) or pigmentation darkness (PC1) and they were therefore not retained in the final models.

DISCUSSION

Here we experimentally tested in a wild bird population how nutritional conditions experienced during the egg laying period affect a female's oxidative status and the allocation of antioxidants into her eggs. Moreover, we tested the hypothesis that protoporphyrin-based eggshell pigmentation signals a female's oxidative condition.

The level of oxidative damage (ROMs) in the female's circulation was strongly influenced by the food treatment. Females that received extra food had lower oxidative damage than non-supplemented females. Components of the antioxidant protection, on the other hand, were not affected by the treatment. One explanation for this finding is that the food supplementation led to a reduction in the production of oxidizing agents. Given that the extra food was provided within the nest, supplemented females may, for example, have reduced their flying and food searching time. Indeed, physical activity can cause an imbalance in the levels of fatty acids, with an increase of unsaturated fatty acids, molecules susceptible to oxidative damage (Porter et al. 1995; Nikolaidis and Mougios 2004). Furthermore, the contraction of skeletal muscles occurring during flying can result in an accelerated production of oxidizing agents, such as superoxide (Powers and Jackson 2008).

In addition, food supplemented females might have had a reduced production of oxidizing agents because of other altered physiological processes, such as an improved immune defence (Catoni et al. 2008b; Costantini and Møller 2009).

Alternatively, the food supplementation might not have reduced the *production* of oxidizing agents, but it may have boosted an (unmeasured) component of the antioxidant machinery (e.g. levels of uric acid or ceruloplasmin; Swennen et al. 2011; Alan and McWilliams 2013). Although the mechanism behind the lower levels of oxidative damage observed in supplemented females remains currently unknown, our result shows that a supplementation of protein-rich food positively affects the oxidative balance of wild-living birds.

Previous studies on captive and wild birds linked the reduction of the ability to neutralize free radicals with an increased reproductive effort. Captive zebra finches (*Taeniopygia guttata*) females, for example, showed reduced red blood cell antioxidant capacity and lower antioxidant enzymes activity when rearing enlarged broods (Alonso-Alvarez et al. 2004; Wiersma et al. 2004). Similarly, great tits rearing an experimentally enlarged brood had a lowered resistance to oxidative stress (Christe et al. 2012). We found that food-supplemented and non-supplemented females had a similar level of reproductive investment (in terms of egg and clutch size), but supplemented females experienced significantly lower levels of oxidative stress. This suggests that the supplementary food helped females to counter the oxidative costs of reproduction.

We found that the antioxidant capacity of the eggs was significantly related to the oxidative status of the mother. Differently from other studies, which observed associations between yolk and female antioxidant capacity (Blount et al. 2002; Butler and McGraw 2013), we found a relationship between yolk antioxidant capacity and female oxidative damage (see also Costantini et al. 2010). Females with higher plasma oxidative damage

levels deposited lower amounts of antioxidants in their eggs, suggesting that oxidative stress may constrain the female's reproductive investment. This negative association between female oxidative damage and egg antioxidant investment was particularly pronounced in non-supplemented females, whereas supplemented females were able to sustain an almost constant deposition of yolk antioxidants, regardless of their circulating levels of oxidative damage. Such a constant antioxidant deposition may be advantageous, because yolk antioxidants can improve embryo development, and a reduction of their deposition may negatively affect the nestlings' survival prospects (Wilson 1997; McGraw et al. 2005).

It has been suggested that females may signal their oxidative condition to the male by modulating the deposition of eggshell pigments, thereby eliciting a higher paternal effort (Moreno and Osorno 2003). However, most studies so far focused on biliverdin-based eggshell colours (Reynolds et al. 2009), whereas the signaling function of protoporphyrin-based eggshell pigmentation is less well understood. In our study we observed a significant relationship between the distribution of protoporphyrin-based pigments on the eggshell and both yolk and female antioxidant capacity. Eggs with high yolk antioxidant capacity and eggs produced by females with high antioxidant capacity (PC1) had more homogenously distributed spots. To the best of our knowledge, this is the first observation of a relationship between female oxidative status and eggshell pigmentation in a species with protoporphyrin-based eggshell coloration.

Male blue tits (*Cyanistes caeruleus*), a species closely related to great tits, were found to adjust their parental effort according to the eggshell pigment distribution (Sanz and Garcia-Navas 2009). It suggests that males are able to discriminate eggshell patterns, also in cavities and nestboxes. Because low nest luminosity favors achromatic signaling (i.e. distribution of pigmented spots) over chromatic signaling (i.e. intensity of spot

coloration) (Aviles 2008), our findings is in line with the hypothesis that females of hole-nesting species signal their condition by modulating the distribution of spots, rather than the coloration of the shell.

In conclusion our results show that (1) food availability experienced during the laying period can profoundly influence the oxidative cost of reproduction in great tit females and, that (2) the female's oxidative status and her nutritional condition influence the deposition of antioxidants into the eggs in an interactive way. Moreover, we found that (3) eggshell spot distribution relates to female and yolk antioxidant status, suggesting an association between the female's ability to cope with oxidative stress and eggshell pigmentation pattern, thereby supporting the hypothesis that eggshell maculation may have a signaling function.

Figure 1. Effect of the food treatment on plasma oxidative damage levels (ROMs; mM H_2O_2 equivalents) in food-supplemented (filled circles; $N = 31$) and non-supplemented (open circles; $N = 39$) female great tits. Means ± 1 SE are shown.

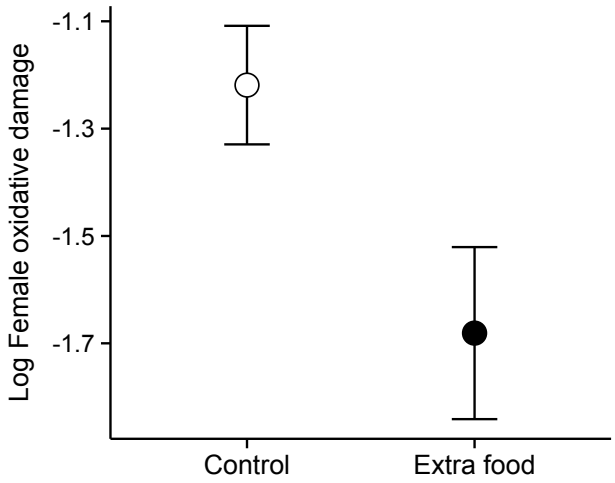


Figure 2. Relationship between female plasma oxidative damage (ROMs; mM H_2O_2 equivalents) and yolk antioxidant capacity (yolk OXY; µM HOCl neutralized mg^{-1} yolk) in food supplemented (filled circles, solid line; $N = 14$) and non-supplemented (open circles, dotted line; $N = 11$) females great tits.

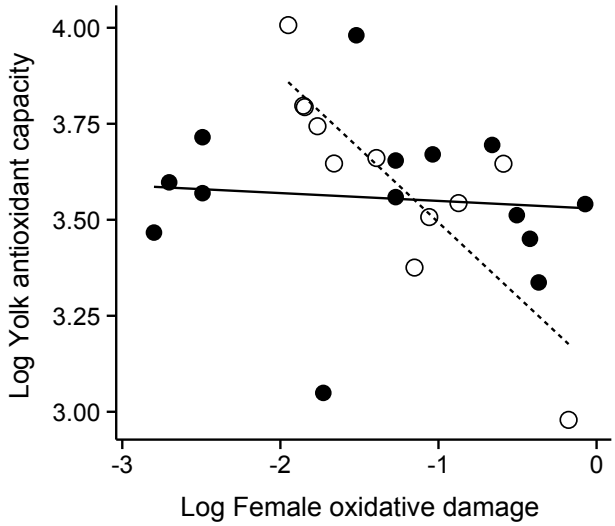
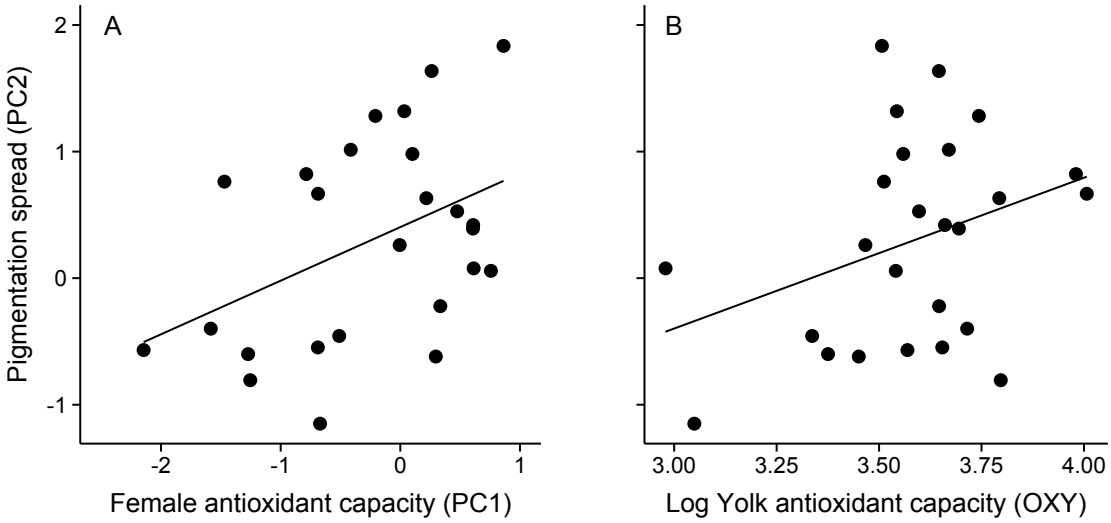


Figure 3. Relationships between eggshell pigmentation (spread (PC2)) and (a) female antioxidant capacity (PC1) and (b) yolk antioxidant capacity (OXY; μM HOCl neutralized mg^{-1} yolk).



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GENERAL DISCUSSION AND CONCLUSIONS

GENERAL DISCUSSION

Over the last twenty years the study of maternal effects became a central research topic for various scientific disciplines. The physiological pathways behind maternal effects and, the ecological and selective pressure driving their evolution have been broadly studied in many species, nevertheless some aspects of these fascinating mechanisms remain still unexplored. The major aim of this thesis was to give new insight into environmentally induced maternal effects and to investigate how a match or a mismatch between maternal and offspring environments influence the morphological and physiological development of offspring.

The work presented in **Chapter one** showed that food-mediated prenatal maternal effects can prime offspring to efficiently use available resources, negating growth-limiting conditions after hatching. These findings highlight how prenatal maternal effects can affect the development of the offspring, with short- and possibly long-term consequences on their fitness. Although previous studies showed that food availability during the egg-laying period can modulate the transfer of yolk androgen into the egg (Verboven et al. 2003; Gasparini et al. 2007), I found no indication of changes of androgen deposition in response to the experimental food supplementation. This indicates that other egg components may have mediated the observed prenatal maternal effects. In agreement with a recent study on captive zebra finches (Krause and Naguib 2014), I did not detect any direct benefit of a match between the pre- and postnatal food availability on nestling morphology. Yet, I found evidence of a positive effect on nestling development of a match of the overall environmental conditions. Given that prenatal maternal effects likely act in response to a wide range of environmental cues, this result highlights the necessity of integrate multiple factors when evaluating the role of environmental predictability in shaping the adaptive value of maternal effects.

The results presented in **Chapter two** demonstrate that the nutritional conditions experienced in the early phases of life play a crucial role in shaping the antioxidant defence of nestlings in a sex-specific way. In birds and mammals, males are often bigger than females, requiring more resources for growth and maintenance and, consequently, being more sensitive to harsh environmental conditions (Clutton-Brock et al. 1985; Anderson et al. 1993). Yet, I showed that female nestlings, even though smaller than male nestlings in great tits, are more sensitive to nutritional stress. It suggests that female and male offspring differ in their sensitivity to maternally derived resources and / or that the two sexes differ in the allocation of resources during early development. The results presented in both **Chapter one** and **Chapter two** show that the morphological and physiological development of nestlings can be mediated by food-induced prenatal maternal effects, and that the nutritional context experience shortly after hatching play an important role in defining the developmental trajectories of nestlings.

Chapter three documented that food availability during reproduction can crucially affect the physiological condition of the breeding females and in turn mediate their ability to deposit resources in their eggs. Oxidative stress has been suggested as a mechanism underlying the cost of reproduction (Costantini 2008; Metcalfe and Monaghan 2013). The results presented in this chapter support this hypothesis, showing that oxidative stress constrained females' ability to transfer resources to their eggs. However, the experimental supplementation with protein-rich food allowed females to maintain low levels of oxidative damage and sustain a constant deposition of antioxidants in the egg, negating the oxidative costs of reproduction. Furthermore, the observed relationship between eggshell spot spread and female antioxidant condition supports the hypothesis that eggshell pattern may signal female ability to resist oxidative stress and it suggests that, in hole-nesting

species, the distribution of shell pigments, rather than the intensity of their colour, may signal female quality.

CONCLUSIONS

The work presented in this dissertation has shed new light on our knowledge of maternal effects and it has opened new challenges for future research. For instance, the evidence of the substantial role of food-induced maternal effects on the development of nestlings, and the absence of a role of hormones behind these processes emphasize the need of other experimental work, in order to identify other possible pathways mediating prenatal maternal effects. The interactive effects of the pre- and postnatal environmental contexts detected on nestlings growth suggests the use of a multifactorial approach in future studies aiming to evaluate the adaptive value of maternal effects in the wild. Moreover, the interactive effects of maternal food availability and oxidative condition on a female's reproductive investment support the suggested pivotal role of oxidative stress in mediating the costs of reproduction, accentuating the necessity of conclusive tests of this hypothesis.

In conclusion, this study is among the firsts testing for the effects of environmental match / mismatch in the wild and integrating the investigation of morphological and physiological aspects of offspring performance. The findings of this study contribute to a better understanding of the effects of local environmental conditions on maternal reproductive investment and offspring performance.

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